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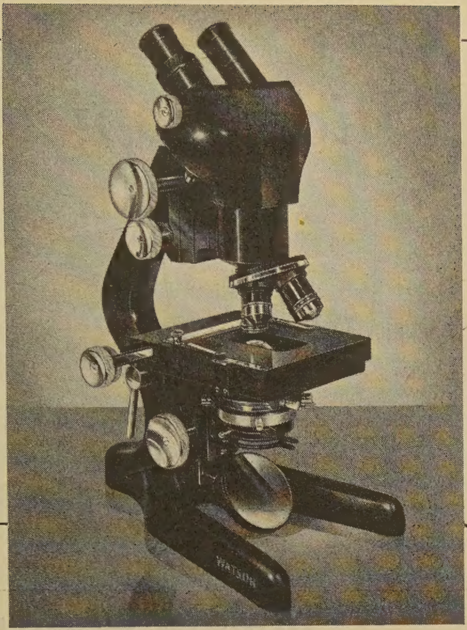

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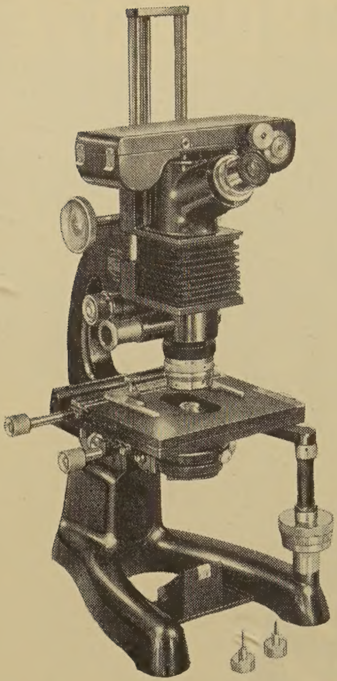

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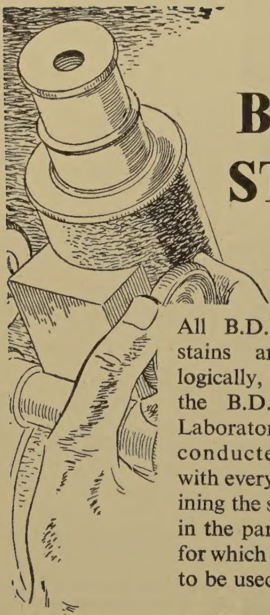

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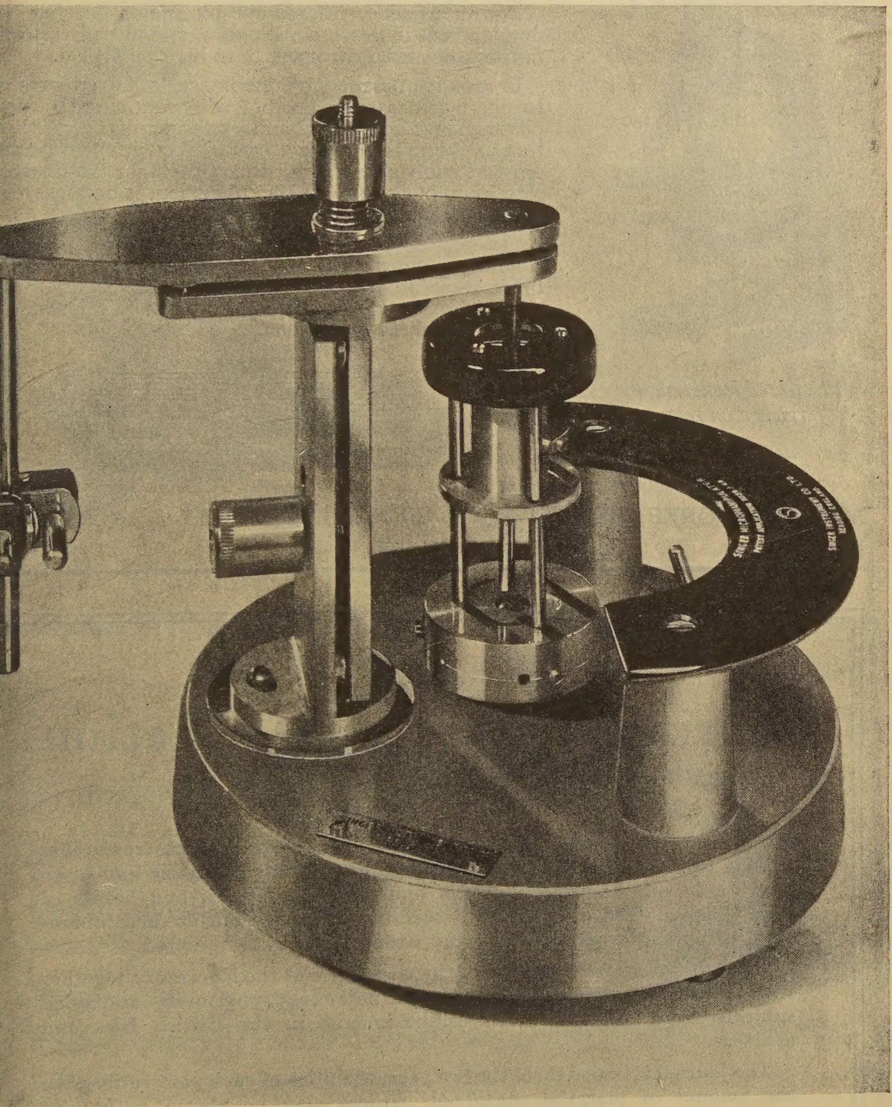

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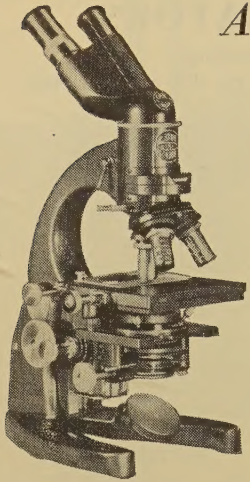

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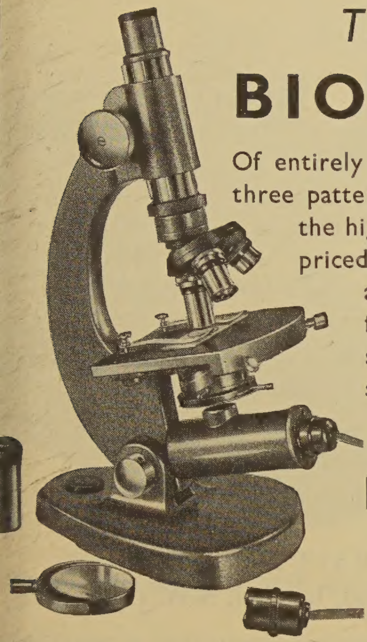

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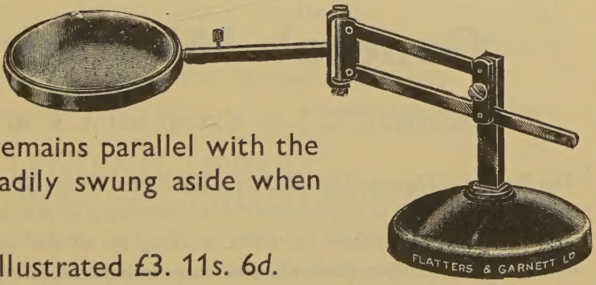

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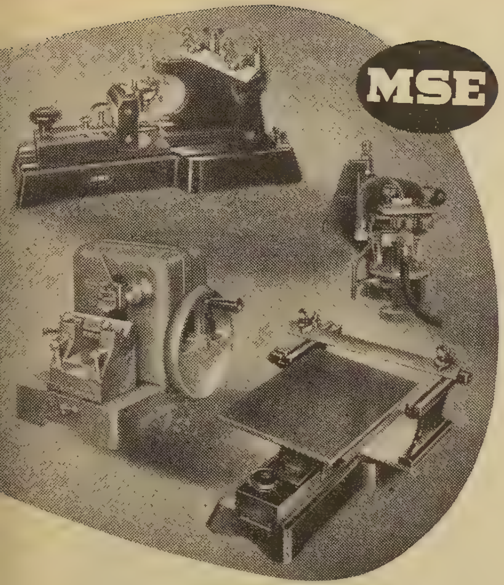

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A Technique for the Histochemical Demonstration of Polyphenol Oxidase and its application to Egg-shell Formation in Helminths and Byssus Formation in *Mytilus*

By J. D. SMYTH

(*From the Dept. of Zoology, Trinity College, Dublin*)

With one plate (fig. 2)

SUMMARY

. The distribution of polyphenol oxidase in quinone-tanning systems may be nonstrated in frozen-dried sections by incubation in 0·2 per cent. aqueous catechol 0° C. for 15–60 minutes. A red colour develops at the enzyme site.

. The evidence for the view that the egg-shell in trematodes, in certain cestode ups, and in turbellarians, is a quinone-tanned protein secreted by the so-called elline' glands, is summarized. The 'vitelline' cells, in addition to giving positive ctions for proteins and phenols, give a strongly positive reaction with the catechol yphenol oxidase test.

. The catechol technique may also be applied to whole helminths fixed in 70 per t. alcohol, and serves as a useful whole mount stain for the shell-producing regions he female genitalia.

. In *Mytilus* the catechol technique reveals the presence of polyphenol oxidase n 'upper' or enzyme gland in the foot.

. It is suggested that in *Mytilus* the byssus is formed from a phenolic protein reted from the phenol gland, which on contact with polyphenol oxidase can dergo 'auto-quinone tanning'.

INTRODUCTION AND PREVIOUS WORK

WITHIN recent years, particular interest has been shown in the structure and formation of quinone-tanned proteins known to occur in various vertebrate groups (Brown, 1950). The basis of quinone-tanning appears to the interaction of a quinone and a protein, the former being formed zymatically from an orthodiphenol in the presence of oxygen (Pryor, 1940). ly in the case of the ootheca of *Blatta* has the diphenol compound been ntified; in this case, Pryor, Russell, and Todd (1946) isolated protocate- uic acid. There is evidence to suggest that, in at least one form, *Mytilus*, diphenol may be an amino-acid or associated with a protein (Brown, 1952). Evidence for the existence of a quinone-tanning system is based on:

(*a*) Macroscopic observation of the actual tanning process on exposure of the material to air.
(*b*) Positive histochemical tests for proteins.
(*c*) Positive histochemical tests for polyphenols.
(*d*) Disappearance of tanning on treatment with sodium hypochlorite.

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on poorly hardened material and was only weakly positive. On the s material, the Sakuguchi and Biuret tests were negative. On theoret grounds, this result was not unexpected as a secretory protein of this kin likely to be in a highly labile and probably soluble state and would easily lost in unhardened material. All these tests proved strongly positive in mate that had been fixed in 5 per cent. formaldehyde-saline, embedded in gelat hardened for 8 weeks in formalin vapour, and cut as frozen sections. Bak elegant modification (1947) of the Sakuguchi arginine test gave a particul strong reaction with gelatine sections. The Biuret test, likewise, gav strongly positive result, the granules appearing rose-pink, a colour indica of lower proteins.

Only in the trematodes have tests for both phenols and proteins b carried out. In the turbellarians and cestodes, only tests for phenols have b carried out and positive results reported—except for a few results for *Polyc* The circumstantial evidence for quinone-tanning in these groups is, howe considerable.

The Catechol Technique for Detection of Polyphenol Oxidase in Tissues

Theoretical basis

The essential reactions in a quinone-tanning system may be summari as follows:

$$\text{protein}+\text{diphenol} \xrightarrow{\text{polyphenol oxidase}} \text{protein}+\text{quinone}$$

$$= \text{tanned protein (sclerotin).}$$

In *Fasciola* rapid auto-tanning of sections does not occur (but see p. 14 so it can be assumed that although the polyphenol, enzyme, and protein c stituents occur in the same globule (fig. 1), some blocking mechanism pr ably prevents the interaction of the phenol and enzyme.

Incubation of tissue containing such a system, with catechol as a substr should result in the formation of *o*-quinone at the sites of the enzyme; quinone should then rapidly combine with and tan the adjacent protein give rise to a reddish-brown colour. A prerequisite of such a technique wo be that the enzyme should be preserved and the protein left still in a 't nable' condition, i.e. with the imino and amino groups more or less int The reddish-brown colour should be very stable and only removable v sodium hypochlorite. The whole reaction should be inhibited by di cyanide.

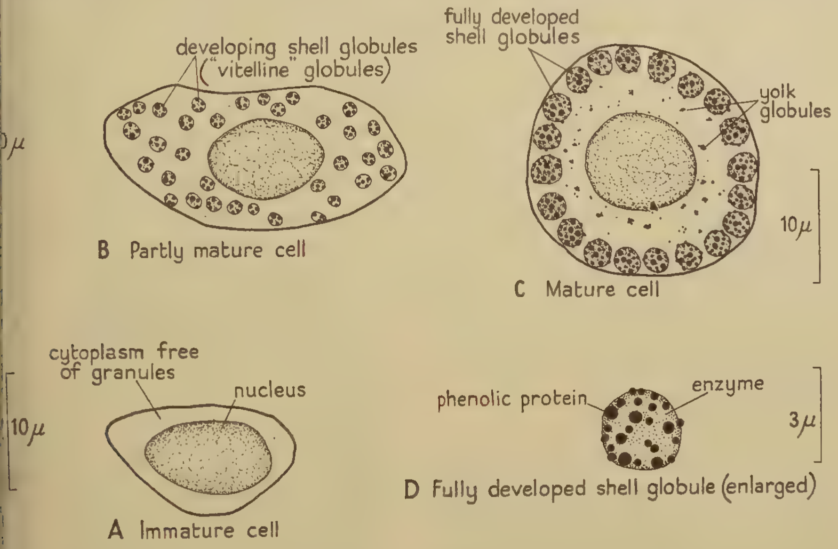
Experimental results

In practice, the method has proved to be remarkably successful. The te nique finally adopted for freshly prepared frozen-dried sections of *Fasc* was as follows:

(1) Remove wax and bring down through alcohols to water.

2) Incubate in 0·2 per cent. catechol (freshly prepared) at 40° C. for 15–30 minutes.
3) Dehydrate, as usual, in 70 per cent., 90 per cent., and absolute.
4) Treat with xylene and mount in balsam.

Vithin 2 minutes of incubation, with this technique, the globules in the lline cells became yellow, and after 10–15 minutes pinkish-red. The lline cells so coloured stood out clearly against the remainder of the tissue 2, B). In sections incubated for long periods (about 12 hours), the vitel- cells became darkish-brown and the remainder of the tissues greyish- wn, the whole almost having the appearance of a haematoxylin-stained tion (fig. 2, F). Since, after several hours' exposure to air, catechol solu- ns become partly oxidized to quinone and this quinone solution will then htly tan any tissues containing protein, irrespectively of the presence of the yme, this result is to be expected. It is essential, therefore, to use freshly pared catechol solution for this test; a solution prepared in the morning y be used throughout the same day, provided it be stored in a reasonably l place. The development of the red colour in *Fasciola* sections was in- ited by the addition of M/1000 KCN to the catechol solution. The red our was also destroyed by 0·1 per cent. NaOCl—both these results being ected on theoretical grounds. Under low-power observation the red colour he vitelline globules appeared sharp, but at oil immersion levels it seldom eared so, a failing shared with the great majority of cytochemical tests olving proteins. The colour occurred only in the larger and mature, or



. 1. Development of the 'vitelline' cells of *Fasciola* as shown in frozen-dried material ned in malachite green and counterstained in Gower's carmine. The large black dots within the shell globules are bright green; nuclei and yolk globules are red.

nearly mature, cells laden with globules of shell material, and not in the sm and immature cells. This result is similar to that obtained with the malach or methyl green techniques; but the red colour, although often havin mottled appearance, was not concentrated within definite granules within globules as is the case with the former technique (fig. 1).

As is shown with *Mytilus*, catechol may form brown condensation-co pounds with phenols, and the dark brown developed in the glands after ab 12 hours in catechol may additionally be due to this reaction. This colour however, almost unaffected by NaOCl.

Effect of storage of material

As work with frozen-dried material progressed, two additional fa emerged: (*a*) that in sections cut and mounted on slides and stored at ro temperature for several weeks, the 'vitelline' globules became slightly tann the deepest colour reached being yellow or pinkish-yellow. This result su gests that slow auto-tanning, which may or may not be enzymatic, can slo take place; (*b*) that at the same time a gradual diminution of the effectiven of the catechol technique took place after prolonged storage—a result int preted as being due to loss of enzyme activity or denaturing of protei or both.

THE CATECHOL TECHNIQUE AS A WHOLE-MOUNT STAIN FOR HELMINTHS

Apart from its use as a histochemical method for demonstration of po phenol oxidase in sections, it has been found that the technique is remarka successful as a whole-mount stain for helminths containing quinone-tann systems.

The simplest and most satisfactory fixative, so far developed for this p pose, is 70 per cent. alcohol, and the procedure adopted for flukes such *Fasciola* and *Dolichosaccus*, and the cestode *Schistocephalus*, has been follows:

(1) Fix in 70 per cent. alcohol—24 hours.
(2) Wash in water—30 minutes.

FIG. 2 (plate). A, *Fasciola* fixed 70 per cent. alcohol and incubated in 0·2 per cent. cate for 90 minutes. Note unusual branching of left 'vitelline' duct.

B, *Fasciola*, frozen-dried section, incubated in catechol solution for 15 minutes. The 'v laria' stand out (red) against the almost colourless cytoplasm.

C, *Fasciola* treated as A.

D, *Schistocephalus*, treated as A.

E, *Mytilus* foot; horizontal section; frozen-dried section stained in malachite green. phenol gland stains green and may be clearly seen to be secreting the byssus through post ducts.

F, *Fasciola* treated as for B, but incubated for 24 hours. Some tanning of cytoplasm has also taken place.

G, *Mytilus* foot; hand section, fixed in 70 per cent. alcohol, incubated in catechol solu for 1 hour.

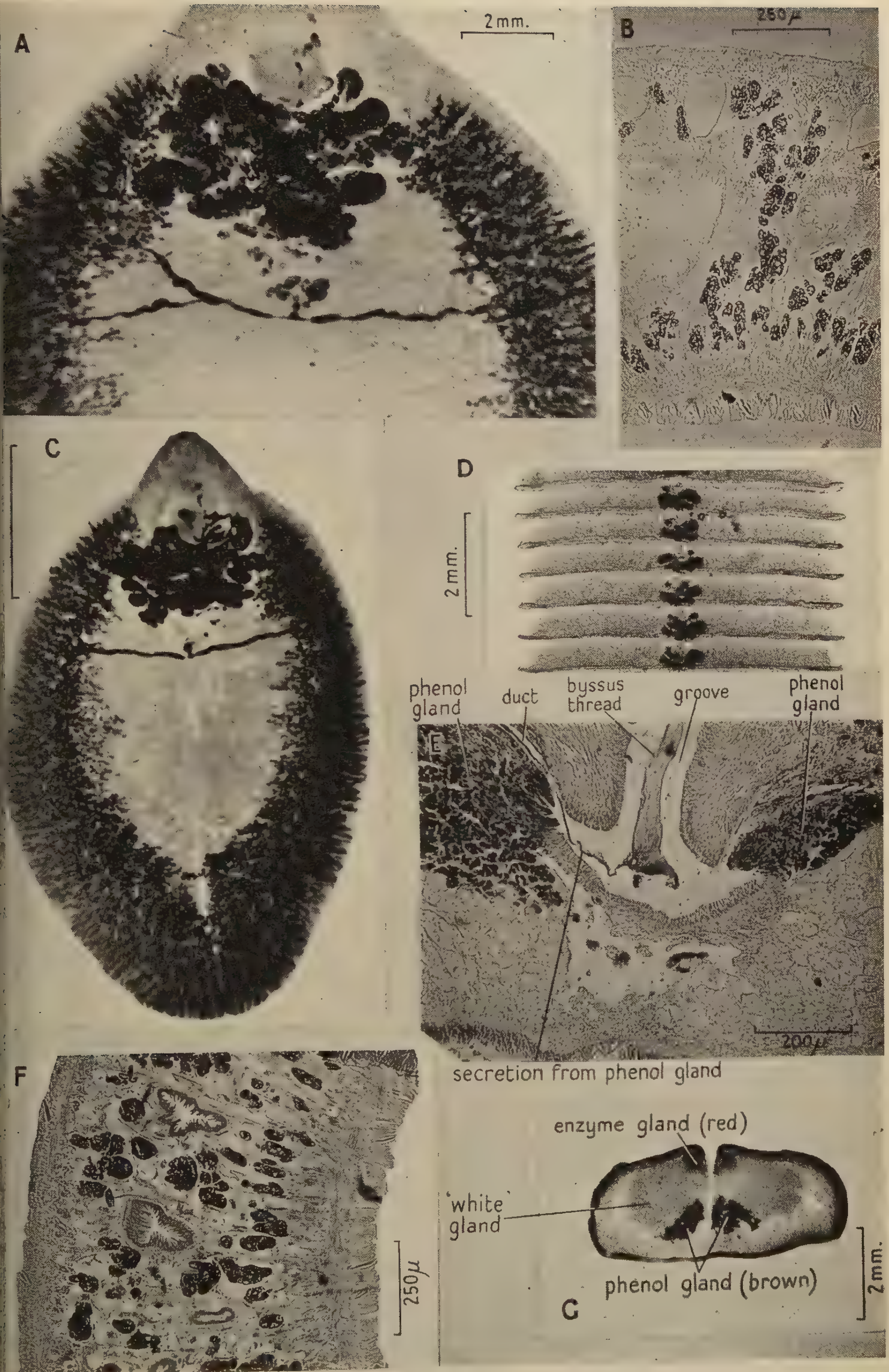


Fig. 2

J. D. SMYTH

(3) Incubate in 0·2 per cent. catechol (Analar, freshly prepared same day)—30–90 minutes.
(4) Wash in water—30 minutes.
(5) Upgrade—70 per cent., 90 per cent., absolute. Pass through xylene and mount in balsam.

xamples of specimens treated by this method are shown in fig. 2, A, C, D.
Fasciola, the 'vitelline' glands, 'vitelline' reservoir, and even the finest
itelline' ducts show up with remarkable clarity. Unexpected branching of
e transverse 'vitelline' ducts are revealed in some specimens (fig. 2, A).
mall flukes, such as *Dolichosaccus*, only require 30 minutes' incubation in
e catechol solution, but *Fasciola* requires about 90 minutes'. The resultant
olour is red to brown, depending on the incubation time. Since the colour
due to the quinone-tanned protein, it is virtually indestructible in any of
e reagents commonly employed in staining techniques; it is, of course,
sily bleached by NaOCl.

After specimens have been 'tanned' in the above manner by catechol treat-
ent, they may be additionally stained (after stage 4) by any of the routine
uclear stains used for whole mounts—borax carmine, paracarmine, &c.—in
rder to stain the remainder of the genitalia not containing enzyme. It is
ecommended that, as a routine, trematodes should be treated with catechol
this way before normal staining.

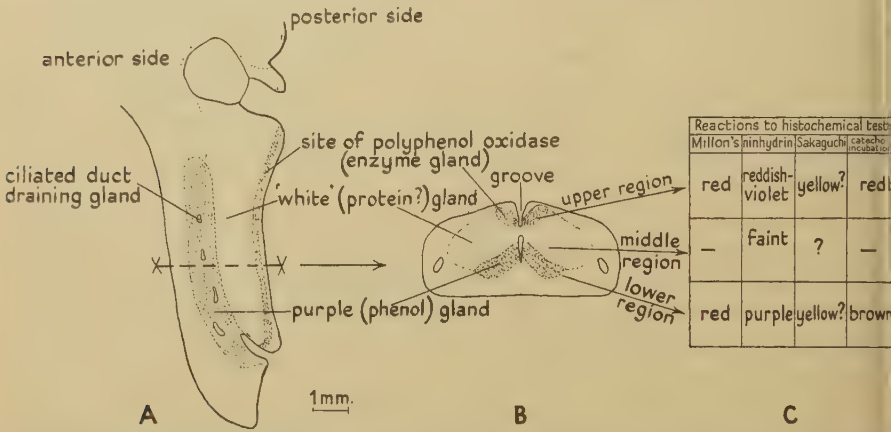
The method may also be used for whole mounts of cestodes (fig. 2, D), but
must be emphasized that it is applicable only to groups forming a quinone-
nned shell similar to that of the trematode egg. The histochemistry of the
estodes is not sufficiently well known, as yet, to define these groups. It has
een found that *Schistocephalus solidus* (Pseudophyllidea) and *Proteocephalus*
licollis (Tetraphyllidea) tan readily by this method, although there is some
vidence to suggest that in *Proteocephalus*, at least, the enzyme is more difficult
preserve than in *Fasciola*. The catechol technique greatly simplifies the
orking out of the histological details of egg-shell formation. Thus, a hel-
inth may be 'tanned' by the catechol technique, dehydrated and treated
ith xylene, and the actual region where the eggs first appear will become
pparent. This region may be cut out, embedded, and sectioned. The shell-
aterial, already reddish in colour, may clearly be seen in sections and the
ytological details of the egg-shell formation worked out.

CONFIRMATORY EXPERIMENTS ON *MYTILUS*

Although the results obtained with *Fasciola* and other helminths were
pparently conclusive, it was considered advisable to test the technique
urther in an organism which was not a helminth and in which a similar
uinone-tanning system was known to occur. *Mytilus edulis* has proved to be
ost satisfactory for this purpose, for its byssus is formed from glandular
issue in the foot by a quinone-tanning process.

A very full account of byssus formation in *Mytilus* has recently been giv by Brown (1952). As results obtained here differ in some points from her a summary of her findings is given below. For previous work on byssu formation, reference may be made to her paper.

According to Brown, the byssus is formed in the posterior groove of th foot from the secretions of two glands: the 'white gland' which suppli protein, and the 'purple' gland which supplies the polyphenol constitue responsible for the tanning; the latter was found to be insoluble in alcoh and there is evidence that it may be a phenolic amino-acid or protein.



Reactions to histochemical test			
Millon's	ninhydrin	Sakaguchi	catecho incubatio
red	reddish-violet	yellow?	red
—	faint	?	—
red	purple	yellow?	brown

FIG. 3. Histochemical reactions of *Mytilus* foot. A is a reproduction of a figure by Brow (1952), as re-interpreted here. B is a transverse section at the point X–X in A. C show histochemical reactions.

Brown believed that the protein material, after being secreted into th groove, is coated on the outside by a layer of phenol which forces itse through the groove epithelium 'to form a border to the mass of white glan material lying in the groove'. Unfortunately, the excellent photomicrograph in her paper are not provided with legends, so that it is difficult to obtain completely clear picture as to her interpretation of the byssus formatio process.

Histochemistry of the foot, and application of catechol technique

Histochemical studies of the foot have revealed the presence of *three* region as seen in transverse section. These are shown in fig. 3. Fig. 3, A, is a re-draw ing of Brown's figure with a re-interpretation. The histochemistry of thes three regions is as follows:

Upper region. After catechol incubation of frozen-dried sections or alcoho fixed hand-sections of the foot, a red region appears on each side of th posterior groove (fig. 2, G). The development of this red colour is inhibite by M/1000 cyanide. The colour is also destroyed by sodium hypochlorit and it is concluded that it represents the site of polyphenol oxidase activit ording to Brown (1952) this region is merely part of the phenol gland eting into the groove. This view is difficult to understand, as there is no ble connexion between it and the phenol gland proper. We agree with her this region gives a positive argentaffin reaction, but it also gives a parti- rly strong and unmistakable reaction with Millon's reagent and also with hydrin. But since polyphenol oxidase is well known to be a copper protein, result is to be expected.

iddle region. This is the 'white' or 'protein' gland of Brown. According er, this gland 'gave all the protein colour reactions but did not give any he polyphenol reactions'. Unfortunately, she did not give details as to the ge of tests covered by this general statement. All the common protein s have been carried out on this region, on both frozen-dried material and tine-embedded material. The Millon test was completely negative: since test works exceptionally well with frozen-dried material, it may definitely concluded that tyrosine is lacking. Of the other protein tests, the Biuret, hydrin, and Sakaguchi tests invariably gave negative results or results ich were questionable. The strongly positive protein reactions given in the e section by the upper and lower glands were never obtained. With the lts on *Fasciola* in mind, the tests were repeated on gelatine-embedded erial hardened in formalin for 2 weeks. Again, the results were consistently indefinite to be considered positive.

ower region. This is the 'purple' or 'phenol' gland of Brown. The results histochemical tests in this region agreed with Brown's except that, again, reen colour was not obtained satisfactorily with ferric chloride, although r subsequent alkaline treatment the red colour was produced. In addition, region gave strongly positive protein tests—the ninhydrin and Millon s giving particularly brilliant reactions. These results thus confirm Brown's w that the polyphenol is a phenolic protein or phenolic amino-acid. After echol incubation, this region became brown (in contrast to the enzyme ion, which coloured red), owing to the condensation of the catechol with phenol. This brown colour was remarkably stable and not easily affected sodium hypochlorite.

ccording to Brown's account, quoted earlier, the material from the phenol nd forces its way through the epithelium of the groove. That this inter- tation is incorrect is evident from fig. 2, G, which is a horizontal section of foot, showing clearly that the phenol gland *opens by posterior ducts* into the ove in the region of the sucker-like depression. It is also evident from this tion that a large amount of material is being secreted by this gland, which gests that it is playing a major role in the formation of the byssus. Further ng the groove the material appears thinned. The interpretation of these ults is discussed later.

EFFECT OF PHENYLMETHANE DYES ON *MYTILUS*

t has been shown that the globules in the 'vitelline' glands of *Fasciola* e a very marked affinity for methyl green, malachite green, and related dyes in neutral solutions, provided that an approved sample of dye is us (Smyth, 1951*a*, 1951*b*, 1953). It was suspected that this phenomenon was some way related to properties inherent in quinone-tanning systems, t most likely explanation being that the dye was held either by the protein the phenol constituent, which, since they occur in the same globule, cou not be distinguished in *Fasciola*. Application of the method to sections *Mytilus* have revealed a similar result. The lower (phenol) gland shows *very marked* affinity for these dyes (fig. 2, E), the middle ('white') gland h no affinity at all, and the upper (enzyme) gland has a slight, but consta affinity. This result has been of considerable use in locating the phenol gla in difficult sections.

DISCUSSION

The catechol technique. From the evidence quoted earlier, i.e. effect KCN, NaOCl, and results of phenol and protein tests, there seems lit doubt that the red colour produced after catechol incubation is indicative oxidase activity of some kind. Since the catechol is oxidized to quinone, it justifiable to term the enzyme a polyphenol oxidase, provided the term is us in a general sense, i.e. merely as a name for an enzyme that will oxid catechol. Such a term would not exclude an enzyme like tyrosinase which also capable of oxidizing diphenols under certain conditions (Sizer, 195 The success of the technique in such widely differing organisms as *Fasci* and *Mytilus* is particularly satisfying and has provided striking experimen confirmation of the soundness of the theoretical background built up previous workers.

Since the colour produced by the catechol technique is due to the tanni of protein molecules, it is clear that it can never *precisely* indicate the enzy site, but only the nearest protein molecule to such a site, although, on the retical grounds, it is likely that the differences between enzyme and prot distribution would be on a molecular rather than a microscopic level. In a case, owing possibly to slight diffusion, the colour under oil immersion never sufficiently intense to allow of more than general localization wit the cell, and the red colour seen under low power is reduced to a rat diffuse apricot colour under oil immersion. For high-power work, then, t technique has its limitations.

Egg-shell formation in helminths. The catechol technique, when applied whole helminths, suggests that a more rational approach to helminth stain may be possible. It also provides a biochemical approach to the problem inter-relationships between helminths, since forms which produce eggs quinone-tanning may be very readily detected. There appears to be a w field here for further investigation, as it is likely that many variations of basic principle of quinone-tanning will occur. For example, it is likely t there are marked differences between such forms as the anaerobic cest *Schistocephalus* and the trematode *Haematoloechus* which lives aerobically the lungs of the frog. Both tan readily in catechol and clearly produce e quinone-tanning processes. Since in *Schistocephalus* matured *in vitro* under robic conditions pre-tanning occurs in the vitelline cells, it may be assumed at normally the phenolic and amino groups and the enzyme are in close ntact, but cannot react during life owing to lack of oxygen. In *Haemato-chus*, on the other hand, presumably some inhibitory mechanism must be veloped to prevent premature tanning, since oxygen from the lung environ-ent would normally be readily available in life.

From the histochemical results described in this paper, together with some npublished observations, some idea of the processes occurring in the so-lled 'vitelline' cell is now beginning to emerge. This is illustrated in fig. 1. the mature cell the shell globules are large and occur almost exclusively the periphery. Since these globules react to both protein and polyphenol sts and redden after catechol incubation, it is clear that the protein, phenol, d oxidase constituents occur in the same globule. None of the histochemical sts for either phenols or proteins has revealed any internal structure to these obules. Yet in frozen-dried sections of *Fasciola* stained in malachite green, is found that instead of the globules showing a uniform affinity for green, e dye is held in large granules clearly visible within the globules. This servation, which was first made on *Fasciola*, has been repeated in several her trematodes with identical results. There is thus also morphological idence for the complex structure of these globules. If, as the evidence from *ytilus* suggests, malachite green is held by protein or phenolic material, ese groups would appear to occur within definite areas of the globules. It ay be that in helminths, as is suggested later for *Mytilus*, the phenol and rotein constituents are the same substance, and the shell substance would us be basically phenolic protein. On this view, the green granules would e phenolic protein and the unstained part of the globules, the oxidase.

Byssus formation in Mytilus. The observations made here suggest that the ccount of byssus formation put forward by Brown may not be correct. ccording to her interpretation, the 'upper' region of the foot (fig. 3, B) is art of the phenol gland. It has been shown here that this region reddens n catechol incubation and is, therefore, the site of polyphenol oxidase. It eems reasonable, therefore, to term this the 'enzyme' gland. Her conclusion hat it was part of the phenol gland is understandable, for near to the point here its ducts open posteriorly into the groove, the phenol gland fills the ulk of the material on each side of the groove, and the 'enzyme' gland, hich is large anteriorly, practically disappears (fig. 4).

Histochemical tests on the lower or phenol gland fully confirm Brown's iew that it is a phenolic protein. If the upper gland is secreting an enzyme nd the lower gland a phenolic protein, what then is the function of the middle land—the so-called 'white' gland? In contradiction to Brown, this region as been found to give only questionable protein reactions, even in material ubjected to prolonged hardening, in contrast to the strong protein reactions f the other two regions. If this region were pouring a protein secretion into he lower part of the groove, strong protein reactions might be expected. Yet the cells of this gland appear to some extent to be orientated towards the low part of the transverse groove, which may indicate that they open into although even in the best preparations an actual secretion in this region h

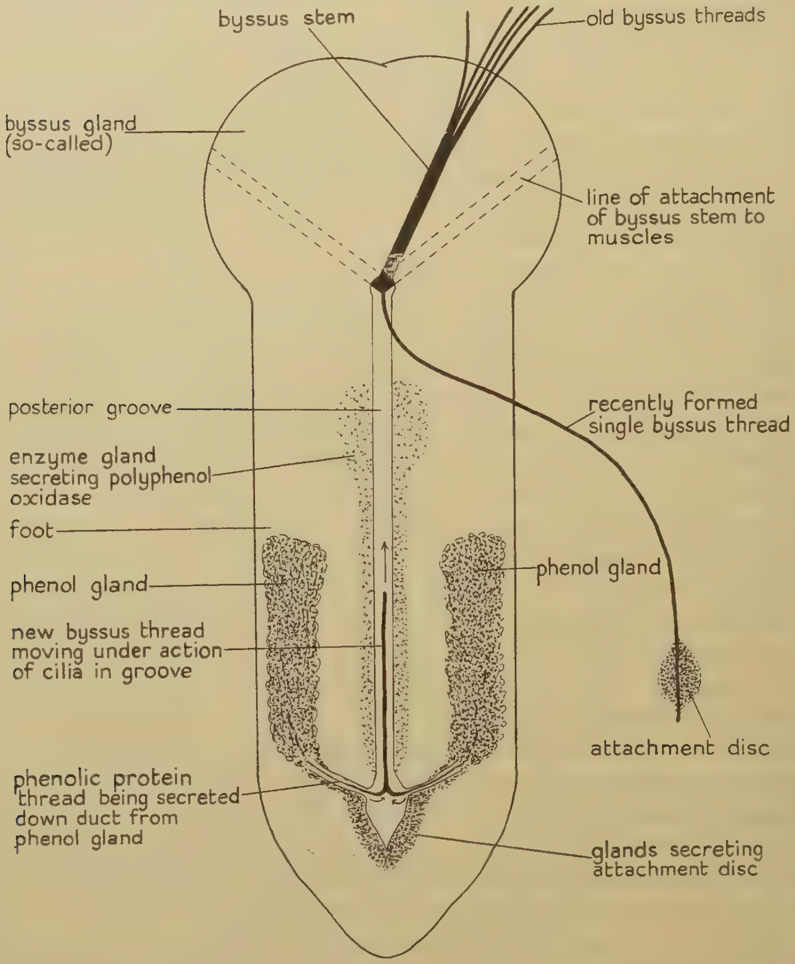

byssus stem
old byssus threads
byssus gland (so-called)
line of attachment of byssus stem to muscles
posterior groove
recently formed single byssus thread
enzyme gland secreting polyphenol oxidase
foot
phenol gland
phenol gland
new byssus thread moving under action of cilia in groove
attachment disc
phenolic protein thread being secreted down duct from phenol gland
glands secreting attachment disc

FIG. 4. Diagram to explain the method of byssus formation in *Mytilus*. The phenol gland open into the distal end of the groove in the foot, into which they pour a thread-like secretio of a phenolic protein. This secretion is drawn forwards along the foot groove by cilary actio and is covered by a secretion of polyphenol oxidase from the enzyme glands on each side the groove. The attachment disk is secreted by cells lining a sucker-line depression near th lower end of the groove. When a byssus thread fills the entire length of the groove, it released except at the end attached to the byssus stem.

never been observed. There would appear to be two possible interpretation of these results. First, the enzyme gland produces polyphenol oxidase, th phenol gland produces phenol, and the 'white' gland produces protein. Apa from overlooking the enzyme gland, this interpretation is close to that of Brow

TABLE I

Results of histochemical tests on the 'vitelline' cells of platyhelminths. + = positive. A blank indicates tests not carried out. Negative results have not been reported

Group	*Organism*	*Worker*	*Tests for phenols*						*Tests for proteins*				
			argentaffin	*chromaffin*	*ferric chloride*	*ammonium molybdate*	*sodium iodate*	*diazo*	*xanthoproteic*	*Sakaguchi*	*ninhydrin*	*Millon*	*Biuret*
Trematoda (Digenea)	*Fasciola hepatica*	Vialli (1933)	+	+	+	+	+	+					
	,, ,,	Stephenson (1946)	+	+	+	+	+		+	+	+	+	
	,, ,,	Smyth (this paper)	+	+	+	+	+		+	+	+	+	+
	Haematoloechus sp.	Smyth (this paper)	+	+	+	+	+		+		+	+	
Trematoda (Monogenea)	*Diclidophora merlangi*	Rennison (1953)	+	+	+	+	+		+	+	+	+	+
Turbellaria	*Dendrocoelum lacteum*	Vialli (1933)	+	+	+	+	+	+					
	,, ,,	Nurse (1950)	+		+								
	Polycelis nigra	Vialli (1933)	+	+	+	+	+	+					
	,, ,,	Smyth (this paper)		+	+	+	+		+			+	
	Gunda segmentata	Vialli (1934)	+	+				+					
	Leptoplana tremallaris	Vialli (1934)	+	+				+					
Cestoda	*Triaenophorus nodulosus*	Vialli (1933)	+	+	+	+	+	+					
	Diphyllobothrium erinacei	Vialli (1933)	+	+	+	+	+	+					

If it were true, the 'protein' produced by the white gland would be endow with peculiar properties, for it is tyrosine-free, extremely labile, and solub This interpretation can be rejected for the reason that a protein sufficient soluble in water to disappear readily in histochemical tests is just as like to go into solution in sea water. Since the byssus remains insoluble, ev before it has become tanned and hardened, this view becomes untenable.

An alternative hypothesis is put forward—that the enzyme gland is pr ducing a polyphenol oxidase and the phenol gland a phenolic protein, t 'white' gland is merely considered to be a developmental stage of the enzym gland. On this view, the bulk of the byssus is secreted from the phenol glan through ducts opening into the distal region of the foot groove. Here t byssus material is wafted forwards and drawn out into fine threads which a moulded by the groove and covered by a secretion of polyphenol oxidase *route* to the so-called byssus gland. When a byssus thread is formed alo the whole length of the foot and an attachment disk secreted, it is release the attachment disk becomes anchored to a rock and the proximal end of t byssus thread firmly bound to the byssus stem embedded in the byssus gla (fig. 4). This view essentially suggests that it is a phenolic protein—*witho any further protein being added*—that under enzyme action becomes tann by the combination of the amino-portion and the quinone nucleus of adj cent molecules. This process might well be termed 'auto-quinone tanning'

This view that the byssus is made up, not of separate phenol and prote constituents, but of one phenolic protein is not unreasonable on theoretic grounds. Brown (1950) has already shown that the product of the phen gland is insoluble in water—exactly the kind of material that would be sui able for secretion into sea water.

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A Histochemical Study of Fat Absorption in the Small Intestine of the Rat

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With one plate (fig. 1)

SUMMARY

Experiments are described in which sections from the small intestine of rats, fed triolein 2¾ hours previously, were examined with Sudan black and the acid haema- n test, with and without Sudan red coloration.

The histochemical appearances in the epithelial cells of the mucous membrane are scribed. Lipoid can be observed not only within the epithelial cells and their free rder but also as particles between the cells.

INTRODUCTION

THE current descriptions of the cellular appearances seen during the absorption of triglyceride fat from the mammalian small intestine state at the fat collects inside the epithelial cells of the mucosa in the region of e Golgi apparatus, and from here it passes down the sides of the nucleus t of the cell. The manner in which it reaches the region of the Golgi ap- ratus is still unknown, but it is generally accepted that the fat traverses the ll on its way through the mucous membrane.

During the absorption of fat it has been shown by Sinclair (1929, 1936), rtom and Peretti (1935), Artom, Sarzana, Perrier, Santangelo, and Segré 937), and others, that phospholipide is formed in the intestinal mucosa and at it contains fatty acids derived from the ingested fat. The role of this ospholipide is debatable, but to it has been ascribed the function of an termediary in the transport of fat through the intestinal mucous membrane. lthough Zilversmit, Chaikoff, and Entenman (1948) had suggested that this ospholipide was not an obligatory intermediate for the passage of fat rough the intestinal wall, it was considered that its formation in the cell ill required explanation. When the experiments described in this com- unication were carried out, they constituted a part of an investigation into e behaviour of the intestinal phospholipide during fat absorption. It soon ecame apparent, however, that the histochemical appearances seen in the ithelial cells were not completely in accordance with the accepted descrip- on, and it is the results of these observations that are presented here.

MATERIAL AND METHODS

The animals used were male albino rats, whose weights ranged from 190 233 grammes and which had been starved for 48 hours but allowed free access

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to water. During their period of starvation they were kept separately in la mesh cages under normal animal house conditions.

Each animal was fed on 225 mg. (0·18 ml.) of triolein by gastric intubat and killed with an overdose of chloroform 2$\frac{3}{4}$ hours later. The quantity of used was chosen because it approximates to the quantity of fat which a would consume in a normal meal.

After death, pieces of small intestine, each 1 cm. long, were removed intervals of 20 cm., starting 5 cm. from the pylorus. Each piece was fixed Baker's (1944) formaldehyde-calcium, after it had been washed through w this fixative.

After the appropriate period of fixation, each piece was subjected to t acid haematein test for phospholipide (Baker, 1946). The technique of t test was carried to the stage when frozen sections 10μ in thickness were c Some of these sections were then treated with a saturated solution of Sud black in 70 per cent. alcohol for the detection of lipoid, while the remain completed the acid haematein test. Finally, some of these acid haemat preparations were treated with an alcoholic solution of Sudan red (Krajian formula).

RESULTS

In general the appearances seen in the Sudan black preparations show t the lipoid content of the cells of the mucosa reaches a maximum in one or t of the pieces of intestine in each animal. Usually there is a progressi increase in lipoid towards this maximal point and a progressive decrease the lipoid in the pieces beyond it. The degree of filling of the cells in the pie maximally filled with lipoid varies from one in which they contain only

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FIG. 1 (plate). Photomicrographs of sections of villi, 10μ in thickness, from the sm intestine of rats.

A, Sudan black preparation. Tip of a villus showing streams of lipoid particles along t intercellular areas, from the free border inwards. A few lipoid particles can be observed in t free border.

B, Sudan black preparation. Side of a villus close to the tip showing cells loaded with lipo The intercellular areas are clear in some places and in others they are obscured by the d tension of the cells with lipoid. Lipoid can be seen streaming down the sides of the nucl to the base of the cell, below the nucleus. Spindles of lipoid can be seen in the free bord

C, Sudan black preparation. Sides of two adjacent villi showing the classical appearances fat absorption. Lipoid can be seen collecting in the region of the Golgi apparatus and strea ing down the sides of the nuclei into the core of the villus. In addition two streams of lip in the intercellular area from the free border inwards can be seen in the left villus, one on e side of the central goblet cell. A third, but less distinct, stream can be seen just below centre goblet cell on the right. Lipoid particles can also be observed in the free border.

D, acid haematein preparation. Tip of a villus showing the localization of the phospholip in the cytoplasm of the cells, with particularly heavy concentration in the bases of the ce below the nuclei. The clear intercellular areas and the clear area below the free border (wh is giving a positive reaction) are to be seen.

E, acid haematein preparation treated with Sudan red. This shows a stream of lip particles overlying the clear intercellular area near the tip of a villus.

F, acid haematein preparation treated with Sudan red. Several streams of lipoid can be s overlying the clear intercellular areas on the side of a villus and some of these extend from free border inwards.

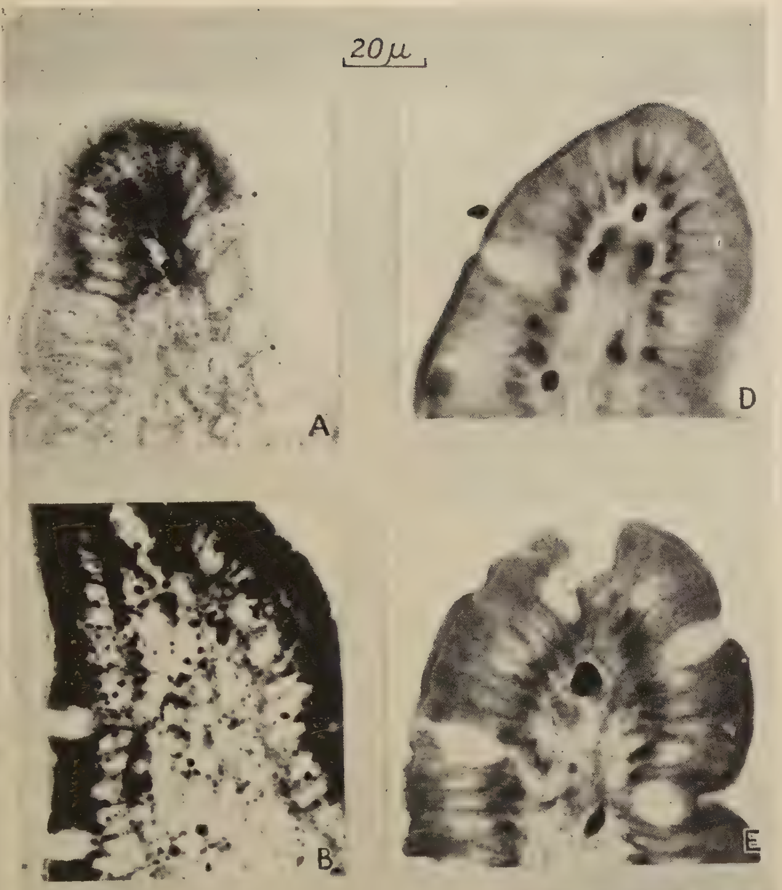

Fig. 1

W. HEWITT

ll quantity of lipoid particles, to one in which they are loaded with lipoid. o, the position this maximally-filled segment of intestine occupies in the ll intestine, as a whole, is not always the same. In some animals it may be first or proximal piece, whilst in others it may approach the centre of the gth of the small intestine.

Vhen the cells are loaded with lipoid (fig. 1, B) the outer part of the cell on lumen side of the nucleus is coloured an intense black by Sudan black. ually between adjacent cells there is an area clear of lipoid, but the cells y be so distended that this clear intercellular space has been obscured. The cosa then presents the appearance of a continuous broad black band. At sides of the nuclei there may be seen a row of lipoid particles that may ount to an uninterrupted stream or just a few isolated particles. The lipoid ectable in the cells below their nuclei is variable in quantity and depends ome extent upon how much lipoid is collecting in this region from that aming down along the sides of the nuclei. When cells loaded with lipoid present, such cells are invariably present at the tips of the villi, and they y occur also at the sides of the villi. The last cells to be involved are those r the base of the villi, but it is uncommon to see these cells loaded with oid. Occasionally, however, these cells contain some lipoid whilst the cells he tip of the villus are quite clear. The phenomenon of loading of the cells h lipoid may, perhaps, be a usual occurrence at the height of fat absorption. was the subject of a paper by Mottram, Cramer, and Drew (1922), and re recently it has received the attention of Frazer (1946, 1948). The latter es that choline chloride hastens the movement of the lipoid into the core he villus when added to the ingested fat.

n those cells which contain only a moderate amount of lipoid, the latter oticed collecting in the region of the Golgi apparatus which also colours ensely with Sudan black. From here a stream of lipoid particles can be erved proceeding down the sides of the nucleus into the core of the villus. addition to these appearances, which are identical with those of current criptions, other lipoid particles can be seen in the intercellular region ending from the free border inwards (fig. 1, A and C). The particles of se intercellular streams of lipoid are frequently very fine and become con- nous with the much larger particles at the sides of the nucleus. It is difficult e certain whether these streams of particles are actually between adjacent s, or whether they are passing along the cell membranes themselves, be- se on some occasions two parallel streams can be observed. This appearance parallel streams may of course be due solely to the fact that one is viewing intercellular region obliquely. These streams of lipoid particles can also be quently observed down the sides of a distended goblet cell. Again, in some tions villi have been accidentally cut obliquely or transversely, and in these, oid particles can be seen scattered around the sides of the outer parts of the s as though they were closely related to the cell membrane.

n addition to these observations some attention has been given to the free striated border of the epithelial cells. The canals in the free border and the appearance of spindle-shaped particles of lipoid in these canals during absorption in the mouse, as observed by Baker (1942 and 1951), can both seen frequently (fig. 1, B and C). The free border is particularly noticea when the cells are loaded with lipoid. Then, not only does it contain spin of lipoid, but lipoid particles can be seen scattered on its surface just as Ba described (1951). The spindles of lipoid are always very obvious when t villi are close to one another with their free borders adjacent, giving impression that the lipoid was prevented from dropping out.

The phospholipide seen in the cells in the acid haematein preparation always surrounded by a clear area between the cells and separated from free border by a similar clear area (fig. 1, D). The quantity of phospholip present can vary considerably, although in its complete absence some min particles can usually be detected at the site of the Golgi apparatus. Wh there may be an even distribution of phospholipide throughout the cytopla of a cell, frequently it is uneven. Sometimes the outer part of the cell conta the most phospholipide, whilst at other times it is the base of the cell be the nucleus which has the greatest concentration. The reaction given by free border may be either negative or positive for phospholipide (fig. 1, D)

The purpose of the acid haematein preparations coloured with Sudan was to demonstrate whether there was any relationship between the locali tion of the phospholipide and the lipoid other than phospholipide in the c In these preparations, however, the most obvious fact was the streams intercellular lipoid particles which could be seen extending from the f border inwards and overlying the clear intercellular region free of phosph pide (fig. 1, E and F). These streams were even clearer than those seen in Sudan black preparations. Also, the spindle-shaped particles of lipoid w easy to observe in the free border, particularly when this was strongly posit for phospholipide. The stream of lipoid particles which collects at the bas the cell from the sides of the nucleus appears to envelop the phospholip collected in the base of the cell.

Discussion

The appearance of lipoid particles in the intercellular region, from the f border inwards, and the possibility that some lipoid is absorbed along t pathway is not a recent suggestion. References to this as a possible rout fat absorption can be found in textbooks published about the beginning of present century. At that time, Kischensky (1902) also described these pearances in a beautifully illustrated paper, and Köster (1908) illustrate section of intestine with streams of lipoid particles extending along the si of the cells. More recently, in the paper previously mentioned, Mottr Cramer, and Drew (1922) illustrate this with a drawing which clearly sh a stream of intercellular lipoid particles from the free border inwards. comment upon this is made in the text of this publication nor by Lovatt Ev (1952), by whom this drawing was reproduced. It is difficult to underst why this conception of fat absorption received so little support, but son appears to lie in the possibility that the intercellular fat had been eezed out of the cells during the course of their histological preparation. s explanation, of course, also applies to the observations reported here. s suggested that the reason for the detection of these intercellular lipoid ticles in the preparations used here depended upon their preservation by previous treatment with potassium dichromate. These particles are very ll and may well be dissolved out in the alcohol of the Sudan black unless jected to treatment with dichromate beforehand. It is interesting to note t Baker (1951) comments upon this action of potassium dichromate in dering detectable the lipoid in the free border. It is, of course, possible t these intercellular particles are diffusion artifacts which have been ected by the technique used and that these experiments should be repeated h the tissues prepared for histological examination by the freezing-drying nnique.

n conclusion, these results appear to demonstrate that, during the absorp- of triolein, not only is it absorbed by passing through the epithelial cells he intestinal mucosa but, in addition, some of it passes between them.

am indebted to Professor R. G. Harrison for originally suggesting this estigation and for his advice and encouragement. I also wish to thank Mr. G. Cooper, Mr. C. T. FitzSimon, Miss B. Birkett, and Miss B. Constantine their technical assistance. The expenses of this investigation were defrayed, part, by a grant from the Medical Research Council.

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A Plastic Lacquer Substitute for Coverslips

By J. S. PERRY

(*From the Laboratory of the Agricultural Research Council, Compton, Berks.*)

SUMMARY

A plastic lacquer manufactured by Cellon Ltd. has been found useful for covering tions on glass slides of any size.

It takes the place of both mounting medium and coverglass and saves storage-space. he method has proved particularly valuable in mounting large series of sections and ial sections of large specimens. Its use in conjunction with bulk staining techniques described. The lacquer is very clear and has not been found to deteriorate on the de after several years.

ERIAL sections or other thin preparations may conveniently be covered with a solution of Cerric lacquer D.010 in Cerric thinner 4977 (three or ur volumes of lacquer to one of thinner). Cerric is manufactured by Messrs. ellon, of Kingston-on-Thames, Surrey; the lacquer is sold at 15*s.* 11*d.* a half- llon, the thinner at 6*s.* a pint. The method has been found both convenient d economical, especially in mounting large series of sections, or serial ctions of large specimens, on glass slides larger than standard size. Quarter- ate 'coverglasses' supplied by Messrs. Ilford have been found to be most itable as slides. They are of the right size and thickness, and of good quality ass. The lacquer can, of course, be applied equally easily to slides of any size d shape, and as it covers the whole area of the glass to the edges, sections n be mounted so as to occupy the whole of the area. A further advantage of is process is that the finished preparations can be stacked on edge like index rds, with no danger of adhering one to another. Considerable saving of time, aterial, and storage space is thus effected.

METHOD

In this laboratory, sections are mounted in the usual way, with Mayer's bumen and water. Special racks have been designed to facilitate the process- g of the quarter-plate slides, which have been adopted as standard for this nd of work. The racks fit into small rectangular museum jars, so that there is tle waste of staining fluids, alcohols, &c. After the sections have been cleared xylene, the excess solvent is drained off, the back of the slide is wiped, and e slide is laid on a flat glass tray. The lacquer solution is poured on from a ottle, the quantity being such that it spreads over the whole area of the slide ithout overflowing. It will be found that this is a simple operation calling for ery little practice. If lacquer is accidentally run over the edge of the slide on the tray, the slide may be lifted or pushed to a dry part of the tray, and re- ooded if necessary. The spilt lacquer may be mopped up, or left to dry and en peeled off.

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When the slides have been flooded, a cover is placed over the tray to prev
dust settling on the lacquer as it dries; it also slows the rate of evaporation a
prevents mottling of the surface, a fault which arises from too rapid dryi
A suitable cover has been made by tacking a double layer of muslin to a shall
wooden frame.

The slides remain under the cover at room temperature for 12–24 hou
after which they may be transferred to a drying oven until the lacquer is qu
hard (24–48 hours). Then the slides can be cleaned and polished. Althou
the lacquer is fairly resistant to accidental scratching, the slides are b
cleaned by washing in warm water with a little detergent, and drying o
soft cloth.

The lacquer fills any scratches on the surface of the glass to which it
applied, so that numbers, &c., written with a diamond, become almost
visible. This may be remedied by cutting and peeling away that part of t
dried lacquer layer which covers the inscription, or by marking the back of t
slide instead of the front, and it is found to be easy to write mirror-ima
letters and numbers so that they are conveniently read through the lacquer a
glass when the face of the slide is upwards.

It has sometimes been found useful to combine this mounting techniq
with bulk staining, the material being stained before sectioning. Bulk staini
with Mayer's haemalum and eosin gives better results than many work
expect, and can well be used when the need is to examine large numbers
organs for particular characteristics. In this case the ribbon is mounted on t
marked slide and, after drying, the wax is dissolved either in xylene or in t
Cerric thinner, the surplus solvent is drained off, and the slides are ready
the application of lacquer. If a particular section proves to be of special
terest, the lacquer can be removed by immersing the slide in the thinner, a
a glass coverslip can then be applied to the required portion of the slide
using a conventional mounting medium such as Canada balsam. If it is desir
to preserve the other sections on the slide, and some are not covered by t
glass slip, lacquer may be applied to the uncovered portion of the slide in t
manner described above.

The lacquer has been found to be of use in covering written labels
museum jars as it forms a perfectly clear, protective veneer.

This Cerric lacquer may be put to a variety of uses in the laboratory, b
many such uses may be served equally well by any one of a number of plas
lacquers. The one described here was selected in a series of trials carried
by the writer with the co-operation of the makers, for the particular purpose
covering preparations for the microscope. Its value for this purpose lies in
miscibility with xylene, and its tolerance of slight degrees of contaminati
with paraffin wax and albumen, as well as in its durability and its firm adh
ence to glass. A quarter-plate 'coverglass' covered with Cerric costs less th
a 3 in. × 1 in. glass slide and a 2 in. × $\frac{7}{8}$ in. glass coverslip. The refractive ind
of the Ilford quarter-plate is about 1·522, and that of the dried lacquer fi
was found to be 1·521–1·522, so that there is little optical interference, a

ly high magnifications can be used. With oil immersion objectives one st select an immersion medium which will not dissolve the lacquer. Wat-’s immersion oil has been found satisfactory and does not appear to affect lacquer, however long it remains in contact. It can be wiped off with lens ue, but the slide is best cleaned by washing, as described above. The lacquer lightly soluble in cedarwood oil, and more readily so in methyl benzoate, that these substances are not suitable immersion media for use with rric’ slides.

Storage of the slides, like index cards, and with actual index cards marking successive series of sections, is of considerable convenience for reference, d a given number of sections thus mounted and stored occupy very much less ce than do conventional preparations. Some slides of this kind have now en stored for more than four years without apparent deterioration.

Much of the work involved in the selection and application of a suitable quer was carried out in the Department of Zoology, University of Bristol, th the skilled co-operation of Mr. Norman Ablett.

he Golgi Apparatus and Lipoidal Bodies in Exocrine and Endocrine Cells in the Pancreas of Man

By DENNIS LACY

rom the Department of Zoology and Comparative Anatomy, St. Bartholomew's Medical College)

With one plate (fig. 1)

SUMMARY

The cells of the pancreas of a woman were studied by means of Aoyama's and aker's methods with the following results:

1. A canal-like Golgi apparatus and lipoidal bodies are observed in both exocrine and endocrine cells.
2. The size of the apparatus and the number of lipoidal bodies are in inverse ratio in both cell types.

hese results do not support the theory that the Golgi apparatus as seen in fixed cells the result of the metallic impregnation of lipoidal bodies.

DURING the past ten years it has been suggested that the classical form of the Golgi apparatus is artificial and results from the distortion of ertain lipoidal bodies during cytological fixation (Baker, 1944, 1949; Thomas, 947, 1948; Foster, 1947; Cain, 1948). This view has not been substantiated y a study of the apparatus in the endocrine and exocrine cells of the pancreas f the mouse (Lacy, 1953). Instead, these cells have been shown to contain *oth* lipoidal bodies and a canal-like Golgi apparatus. The present investigaion had a twofold function:

1. To observe whether both canals and lipoidal bodies are present in the fixed pancreatic exocrine and endocrine cells of man, and
2. To examine further the Golgi artifact theory in the above cells.

MATERIAL AND METHODS

The material became available when a simple splenectomy was carried out n a woman aged 54, after hypersplenism. This condition was secondary to ortal obstruction which was due to primary carcinoma of the liver. The atient had a small breakfast at about 8.45 a.m. and was operated on at 1.30 p.m. During this time no stimulants such as adrenalin or glucose were given. Immediately after its removal from the patient the pancreatic tissue was placed in formaldehyde-saline. Ten minutes later the tissue was cut into six small pieces, each about 3 mm. square. Four pieces were left in the

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formaldehyde-saline and the remaining two pieces were placed in Aoyam fixative. The material was then treated by the following methods:

Aoyama's method for the Golgi apparatus with post-chroming

Fix in Aoyama's fluid for 4 hours and rinse quickly with distilled wat Place in 1½ per cent. solution of silver nitrate for 19 hours and rinse agai Then place in Aoyama's reducer for 5 hours. Wash the tissue for 1 hour, a place it in 5 per cent. potassium dichromate for 24 hours at room temperatu and then for a further 24 hours at 60° C. Wash for 24 hours and embed paraffin wax.

Baker's (1949) *method for lipoidal bodies*

Fix 1 hour in formaldehyde-saline and then 5 hours in dichromate-form next for 18 hours in 5 per cent. potassium dichromate at room temperatu and finally 24 hours at 60° C. Wash for 24 hours and embed in gelati Colour sections with Sudan black and stain with carmalum.

RESULTS

Acinous cells

Aoyama's method (with post-chroming) revealed a large black Golgi ne work in most of the cells (fig. 1, A, C, and D). The network lay in three plan passing around and amongst the zymogen granules. The nets consisted of can like structures in which the walls were more heavily impregnated than t lumina. The canalicular form was particularly distinct when the cells we viewed under phase-contrast. The canals were wider and more numero than those seen in the mouse.

Baker's method. Unstained canalicular structures, passing around a amongst the zymogen granules, were clearly visible in dichromate-formald hyde/Sudan black sections (fig. 1, E). These could be seen with particul clarity by means of phase-contrast. Their shape, size, numbers, and distrib

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FIG. 1. A, a photomicrograph by ordinary transmitted light of the Golgi apparatus exocrine and endocrine cells of the mouse pancreas (Aoyama's method, with post-chromin The apparatus is hypertrophied in the exocrine cells and atrophied in the endocrine cells.

B, photomicrograph by ordinary transmitted light of the lipoidal bodies of exocrine a endocrine cells of the mouse pancreas (Baker's dichromate-formaldehyde/Sudan bl method). There are masses of the lipoidal bodies in the endocrine cells and particularly in single endocrine cell amongst the acinous tissue. There are few such bodies in the acin cells. The Golgi apparatus is not visible in either cell type (see E below).

C, a phase-contrast photomicrograph of two acinous cells showing the canal-like struct of the Golgi apparatus (Aoyama's method, with post-chroming).

D, a diagram of C, emphasizing the canalicular form of the Golgi apparatus. The broken l outlines a nucleus and part of the Golgi apparatus at different foci.

E, a phase-contrast photomicrograph showing the unstained canal-like apparatus in t acinous cells (Baker's dichromate-formaldehyde/Sudan black method). Some lipoidal bod are seen on the canals. This phase-contrast photomicrograph should be compared w photomicrograph B, in which the canals are not visible by ordinary transmitted light.

F, photomicrograph by ordinary transmitted light of some lipoidal bodies in islet ce Their vacuolated and 'capped' form is seen quite clearly.

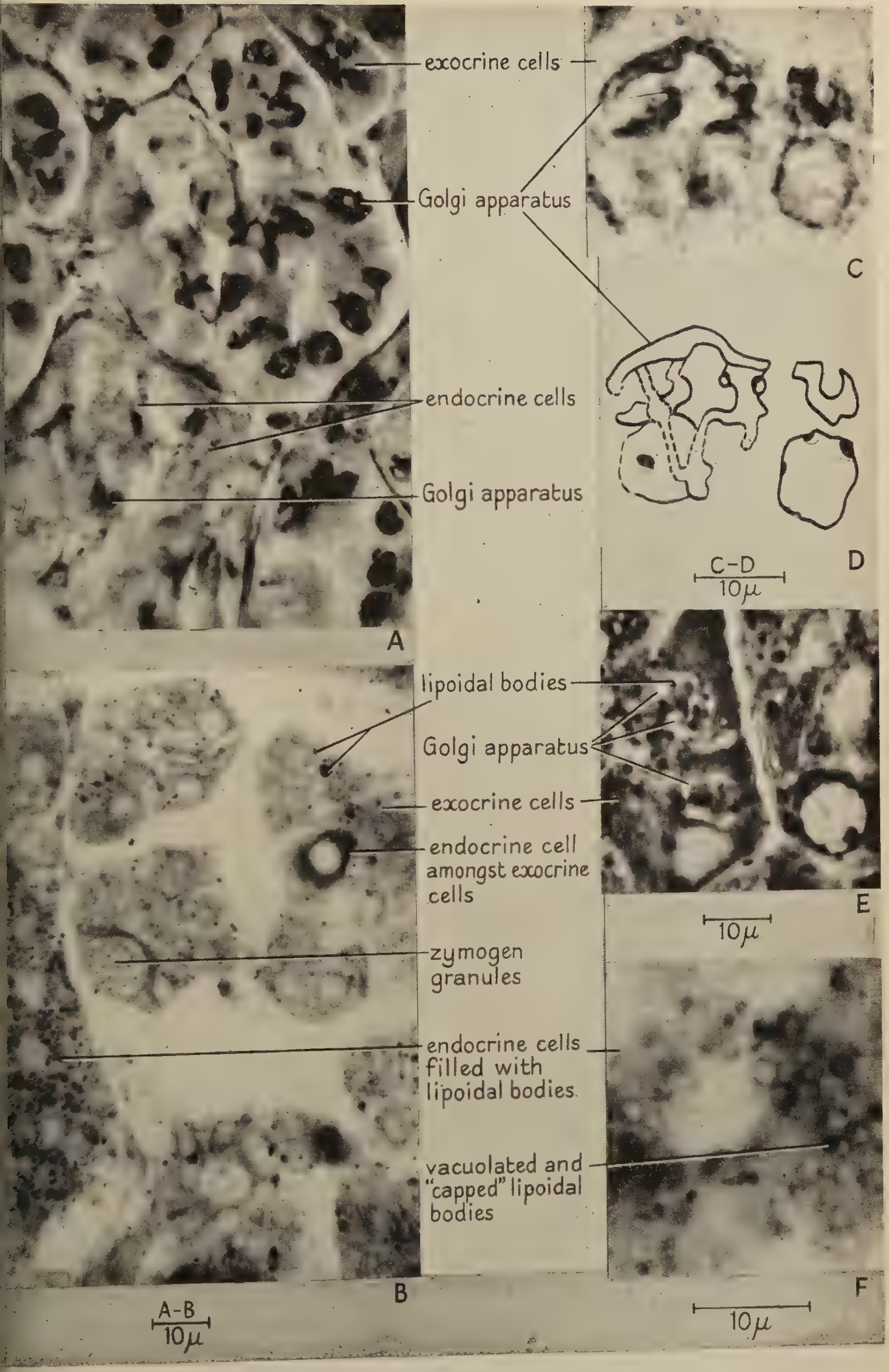
exocrine cells
Golgi apparatus
endocrine cells
Golgi apparatus
A
C
D
C-D
10μ
lipoidal bodies
Golgi apparatus
exocrine cells
endocrine cell amongst exocrine cells
E
10μ
zymogen granules
endocrine cells filled with lipoidal bodies
vacuolated and "capped" lipoidal bodies
B
A-B
10μ
F
10μ

Fig. 1

D. LACY

n left no doubt that they were similar to the canals shown in silver by the thod of Aoyama. Some of the canals showed unstained striae, a result nd previously in the acinous cells of the mouse.

Few lipoidal bodies were present (fig. 1, B). These were generally granular d in contact with canals (fig. 1, E). The cells were full of zymogen granules.

et cells

Aoyama's method. In many cells there was either no silver or else only a all granular deposit (fig. 1, A). In a few cells the silver had impregnated a all strand-like or filamentous structure. The general appearance of these ls suggested that there had been a failure in technique. However, this emed scarcely possible since the Golgi apparatus was clearly present in the rrounding acinous cells. The most reasonable explanation may be that the ver in the islet cells impregnated a Golgi apparatus in various stages of ophy.

Baker's method. Sections were examined by phase-contrast and by ordinary nsmitted light. In a few cells there was an unstained canal-like structure, rresponding in shape, size, and position to the filamentous structure (Golgi paratus) shown by the use of the silver technique. There seems no doubt at the Golgi apparatus of these cells is a canal-like structure similar to that entified in islet cells of the mouse.

Sudan black revealed an extraordinary number of lipoidal bodies in almost ery cell (fig. 1, B). The majority of these were large and vacuolar (fig. 1, F). ranular and multi-vacuolated forms were also observed. Many of the cells emselves were degranulated and vacuolated.

These results were particularly noticeable in single islet cells found amongst inous tissue (fig. 1, B).

Discussion

It would appear that in both acinous and islet cells of the pancreas of man ere exists a canal-like Golgi apparatus and lipoidal bodies as well. Further, e results here obtained allow a re-examination of the view that the classical rm of the Golgi apparatus is the result of artificial over-impregnation of poidal bodies.

In the acinous cells there were few lipoidal bodies, most of which were ranular. Yet a classical silver method and Baker's modern method revealed large canalicular Golgi apparatus. In the islet cells, on the other hand, there ere masses of lipoidal bodies, most of which were vacuolated, while the olgi apparatus was absent or atrophied. Now, if the view is accepted that, fter the use of a classical method, the Golgi apparatus is formed by the dis- ortion of lipoidal bodies (and one would imagine, particularly *vacuolated* odies), then the apparatus should be diminutive in the acinous and enormous the islets. Precisely the opposite was found. Therefore, the present artifact ypothesis of Baker and others is rejected. The evidence presented here, of ourse, cannot mean that a canal-like Golgi apparatus necessarily exists in fe. This question has, however, been decided in the affirmative in the case of the endocrine cells by the examination of living material (Bensley, 191 O'Leary, 1930; Beams, 1930; Lacy, in the press). Certainly, there is no dou that in fixed cells the Golgi apparatus and lipoidal bodies are morphologica distinct organelles.

Previous work on the lipoidal bodies and Golgi apparatus of both pancrea exocrine, endocrine, and other cells has suggested that:

1. The Golgi apparatus undergoes changes in size and structure duri the formation of secretory granules (Duthie, 1933; Sluiter, 1948; La 1953).
2. The lipoidal bodies arise either on the Golgi apparatus or may pa towards it (Moussa, 1950, 1952; Lacy, 1953).
3. The lipoidal bodies are at first granular, then develop a vacuole, a next erupt and liberate their contents as secretory granules (Bak 1944, 1949; Thomas, 1948; Lacy, 1953).

In the acinous cells of the patient reported upon there were masses zymogen granules, few lipoidal bodies (most of which were granular) and hypertrophied Golgi apparatus. This suggests that the cells were in the re tively inactive storage phase. This view is supported by the fact that t patient had eaten only two light meals during the 22½ hours preceding t splenectomy, one at 4 p.m. the previous afternoon and the other at 8.30 a. the following morning.

The islet cells showed much degranulation and vacuolation. There we masses of lipoidal bodies, most of which were vacuolated and some ev multi-vacuolated. The Golgi apparatus was absent in many cells, while others it was present only as a small canal-like structure. The degranulati and vacuolation suggests that the cells had passed through an exhausti secretory phase (Woerner, 1938, 1939; Latta and Harvey, 1942). The mass of lipoidal bodies suggest that this phase may have been followed by the p paratory stages of islet-granule synthesis as evidenced in 3 above, for it m be recalled that, with reference to exocrine cells, Baker (1944) has state 'It is clear that there has been a great synthesis of lipoid, preparatory to t synthesis of zymogen.' The atrophy of the Golgi apparatus described abo may perhaps be reconciled with the view that it has become exhausted af the secretion of its contiguous lipoidal bodies (see 2 above).

We have seen that the islet cells were in an unusual condition, but we cann at present state whether the abnormal condition of the patient (carcinon hypersplenism, &c.) or the operative treatment (involving anaesthes surgical shock, and possible other factors) was responsible.

My most grateful thanks are due to Dr. A. J. Marshall for suggesting t research, and to Mr. A. H. Hunt, F.R.C.S., who kindly provided the materi I should also like to thank Mr. C. Boswell for helpful discussion and Miss Jeremy for technical assistance.

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the Role of the Integument in Acarine Development and its Bearing on Pupa-formation

By BRYN M. JONES

(*From the Department of Zoology, University of Edinburgh*)

With one plate (fig. 7)

SUMMARY

Typical development in mites begins with the retraction of the epidermis from the ticle. It is accompanied by a decrease in size and a change in shape. Atypical de- opment also begins with a moult. But it is distinguished by regrowth, a change shape, and an extra moult.

Each moult is unique in that the epidermis divides into an outer and an inner layer. e outer layer adheres to the old cuticle. Its cells are provisionally regarded as being source of the moulting enzymes. The inner layer retracts and persists as the e epidermis.

The components of mite cuticle, according to histochemical tests, resemble those insect cuticle.

The formation of a provisional cuticle during atypical development produces the ndition generally recognized as a pupal state. The moult of this provisional cuticle is imarily aimed towards synchronizing cuticle deposition with muscle development. tegration of the intermediary connecting processes, the fibrillae, with both the uscle ends and the cuticle is thus made possible.

INTRODUCTION

THE main purpose of this communication is to describe the unique role of the acarine epidermis during moulting, and to define the essential ature of pupa-formation on the basis of observations on acarine development.

The structure of the arthropod integument and the process of moulting ave been described in the decapod Crustacean (*Homarus*) by Yonge (1932), the Insecta (*Rhodnius* and *Tenebrio*) by Wigglesworth (1933, 1948), and the Araneida (*Tegenaria*) by Browning (1942). Lees (1947) pointed out that e structure of the cuticle of a tick resembled that of an insect. However, ttention had not hitherto been properly focused upon either the degree of ariation or the process of moulting in acarine development.

It so happens that some mites in their development produce a pupal con- ition. In view of this it is somewhat surprising that no suggestion, as far as am aware, had been made, that a clearer understanding of the pupal state n arthropods might be elicited from an investigation of this condition in mites. he beautifully executed figures of *Trombidium fuliginosum* by Henking (1882) ave a reasonable clue. However, it was the close resemblance between the upal state expressed in the development of the British harvest-mite (Jones, 951) and that of insects which led to part of the present work.

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MATERIAL AND METHODS

A tyroglyphid mite *Histiostoma polypori*, an oribatid mite *Scheloribates lae* *gatus*, and the British harvest-mite *Trombicula autumnalis* were used for stu A method for rearing mites has been described (Jones, 1951). Much of t success of sectioning these minute animals with their sometimes extreme hardened cuticle depended upon a judicious use of the microtome blade. H alcoholic Bouin was used for fixation, and the hardening effects of absolu alcohol and xylene were eliminated by substituting amyl acetate. Paraffin w of m.p. 56° C. or Peterfi's celloidin-paraffin was used for embedding. Mayer haemalum, Heidenhain's haematoxylin, and eosin were the principal stai used.

THE INTERPOSED PHASES OF DEVELOPMENT

It seems reasonable to suggest that mites fall into two natural groups accor ing to their development. In one group each phase of development is accom panied by a typical single moult. In the other, each phase of development punctuated by two moults. The extra moult makes it atypical. It also involv the condition generally recognized as pupa-formation. Each atypical phase development therefore assumes a pupal state (Jones, 1950).

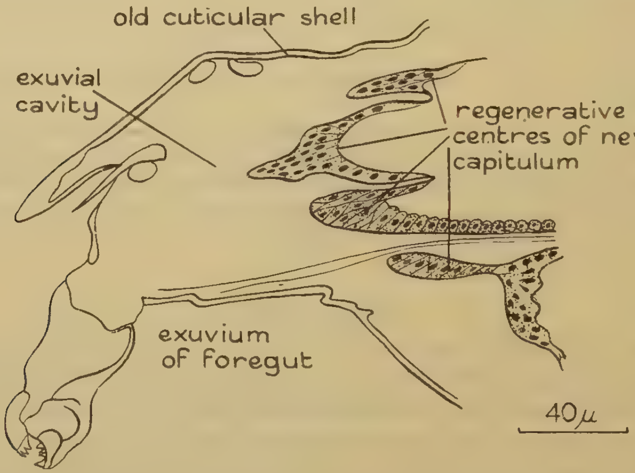
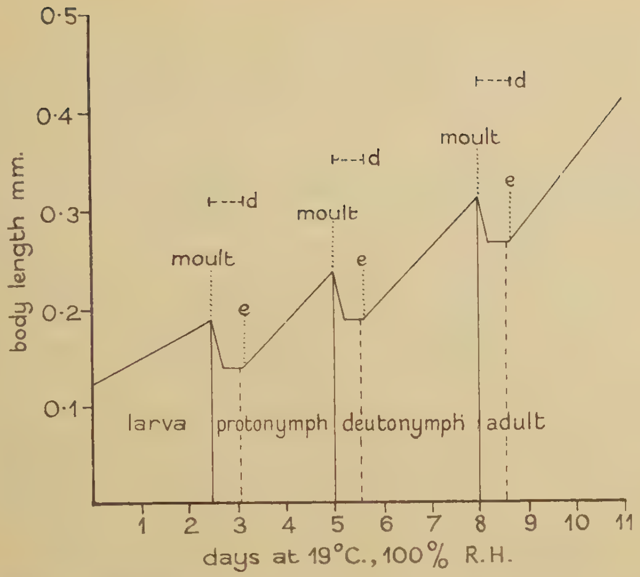


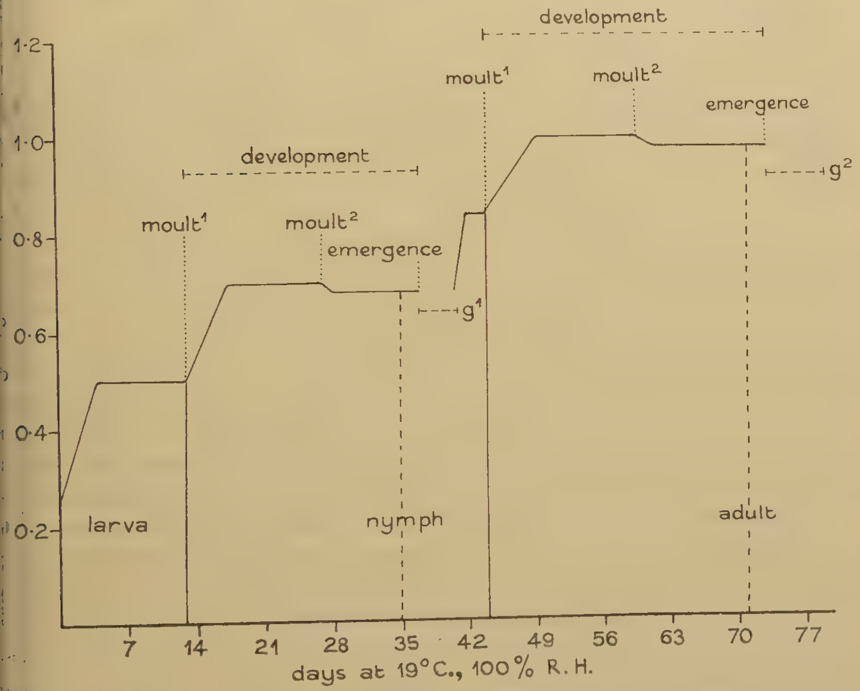
FIG. 1. Section of a typically developing mite showing the spaciousness of the exuvial cavi and the epidermal regenerative centres moulding the capitulum of the next stage.

Of the mites examined in this work, the tyroglyphid and oribatid mit develop typically. Each phase of development is initiated by the retraction the epidermis from the cuticle. The retraction is considerable. The mite n only shows a marked decrease in size, but also a change in its spatial relatio (fig. 1).

The first instar or hexapod stage is conveniently referred to as the larv The succeeding stages (the number varies with the species) which interve before metamorphosis to the adult are termed nymphs. A stage has two we



2. A diagram illustrating periodic growth in a mite, *Histiostoma polypori*, which has cal developmental phases. d, phase of development; e, emergence from cuticle of previous stage.



3. A diagram illustrating periodic growth in a mite, *Trombicula autumnalis*, which has pical developmental phases, each being distinguished by a moult of provisional cuticle. nd g^2 indicate the free-living existences of the nymph and adult respectively; in nature they nay extend considerably to make the life-span from egg to adult last about 6 months.

defined phases: one the immobile developmental phase; the other, the mol growth phase. Any recognizable advance or change in development may b be termed a step.

In general, it is convenient to regard the moulting cycle as beginning w the retraction of the epidermis from the cuticle. It continues with the c solution of the separated cuticle. The discarding of the exuvium is mer incidental to emergence which often takes place some time after the moult

As shown in fig. 2, the retraction of the epidermis from the cuticle *Histiostoma polypori* marks the end of a stage and the initiation of the ne In fig. 3 the beginning of a stage in *Trombicula autumnalis* is likewise mark by the retraction of the epidermis from the cuticle. However, the succeedi phase of development is further punctuated by a second moult. Because t occurs the mite assumes a pupal condition (fig. 7). It also became appar that regrowth (fig. 3) and a change of shape accompanied pupa-formation.

The Component Layers of Mite Cuticle

The results of histochemical tests on the lines devised by Wiggleswor (1933) demonstrated that the nature of the cuticles of the mites chosen study resembled that of a tick (Lees, 1947) and that of insects (Wiggleswor 1933, 1948).

The epicuticle of *T. autumnalis* is a uniform limiting membrane, which amber in colour. It is less than 1μ thick. When this mite was pretreated wi cold chloroform and immersed in silver hydroxide solution the entire surfa of the cuticle stained brown. The argentaffin reaction demonstrated that 'polyphenol' layer had been exposed after treatment with the cold chlorofor Cold chloroform is capable of dissolving away an outer layer of wax provid a 'cement layer' is not present. Treatment with hot caustic potash first di solved the endocuticle before leaving finally a layer which broke up into o droplets. This layer of the epicuticle termed the cuticulin layer has been pr visionally regarded as consisting of a lipo-protein (Wigglesworth, 1948). was therefore evident that the epicuticle of this mite was composed of a w layer, a polyphenol layer, and a cuticulin layer.

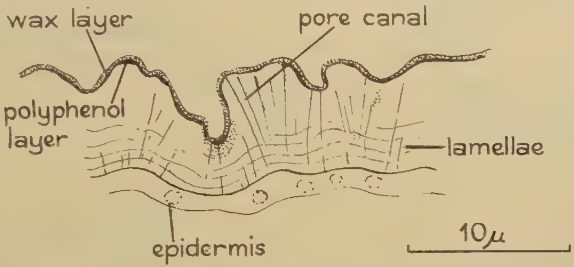
The epicuticles of both tyroglyphid and oribatid mites could be readi differentiated histologically into two layers. In sections treated with haem toxylin and eosin the sclerotized exocuticle stained reddish. But the cuticle certain parts such as the capitulum, the legs, the genital plates, the poro plates of large dermal glands, and the bases of setae has no affinity for stai It retains its natural uniform amber colour. These amber-coloured regions fact represent the hardest parts of the general cuticle.

The epicuticle of these mites, like that of the harvest-mite, was compos of an outer wax layer, a polyphenol layer, and a cuticulin layer according the tests already mentioned.

The lamellated structure of the endocuticle was best seen in sections of the mites either before, or after, they had emerged. Sections of cuticle in w ted first with xylene before being immersed in silver solution demonted the presence of delicate pore canals (fig. 4).

The provisional cuticles of *T. autumnalis* formed during each phase of deopment possessed, according to the tests described, a true epicuticle. They re also resistant to concentrated acids and alkalis, another property of the cuticle.

Before the emergence of either a tyroglyphid or an oribatid mite from its old icle or exuvium, the exocuticular region of the new cuticle was already ablished and tanned. A part of the inner soft endocuticle had also been laid wn. The muscles became effectively attached to the cuticle at this time. ost of the endocuticle was secreted after emergence.



. 4. Section of cuticle of a mite before emergence, stained with silver hydroxide solution.

On the basis of these tests the cuticles of the mites studied were each mposed of two essential layers, namely, a thin outer epicuticle, and a relatively ck inner layer, the endocuticle, capable of becoming tanned on the outside to m an exocuticle.

Changes in the Integument during Moulting

The only cellular elements of the epidermis other than the sensilla, which y be regarded as complex, are the dermal glands. In the harvest-mite *T. tumnalis* a pair of obscure dermal glands are situated midway along the rso-lateral surface of the idiosoma. They are relatively large sac-like structres having a neck region and they protrude well below the epidermal layer. ne contents consist of spheres of acidophil cytoplasm and indistinct nuclei. *Scheloribates laevigatus* there are three kinds of large dermal glands. Eight irs of multiple glands in the nymph are arranged along the dorso-lateral gion of the idiosoma. Each communicates with a sunken multi-perforated al plate, and each pore is connected by a duct to one of the large pear-shaped ls with its acidophil cytoplasm, a large nucleus, and usually a vacuole. ne mass of closely packed pear-shaped cells projects some way below the idermis into the haemocoel (fig. 5, F). In the nymph these multiple glands, th their sunken porous plate and accompanying seta, closely resemble the pit ands of *Tenebrio* (Wigglesworth, 1948). In the adult, a relatively deep pit th porous walls is substituted for the plate.

In the same plane as the multiple glands, situated posteriorly, is a pai dermal glands of quite different appearance which may be termed the bro glands, because on deterioration they produce a brown exudate. The glanc oval, and consists of large cells, with acidophil cytoplasm, and weakly basip nuclei, which surround a cuticle-lined lumen communicating with the exter through a pore of a raised tubercle (fig. 5, G). A pair of contiguous derm glands are closely pressed against the anterior face of the perforated cuti of the body.

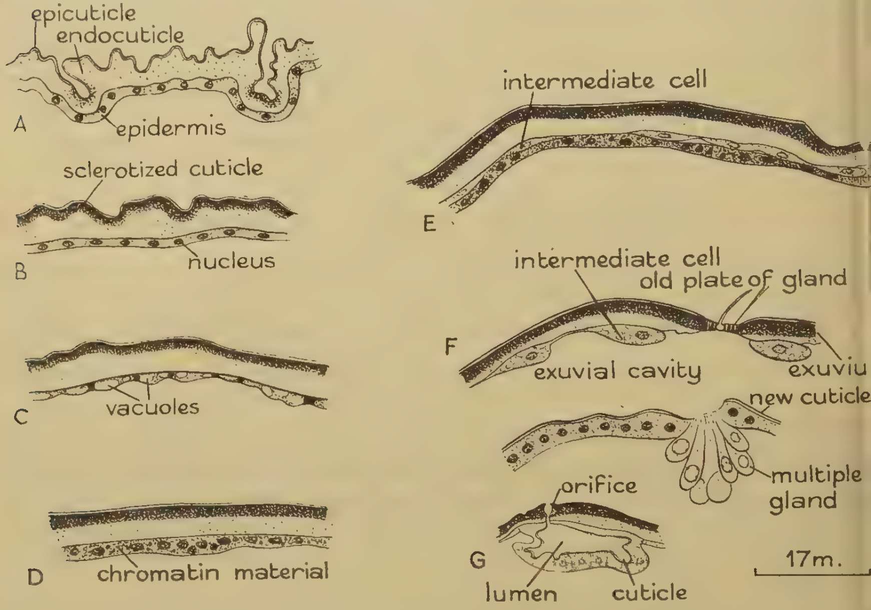


FIG. 5. Changes in the integument of the final nymph of an oribatid mite from the time emergence to the metamorphic moult. A, pre-emergence; B, early growth phase; C, m growth phase; D, late-growth phase; E, beginning of moult showing the epidermis in process of dividing; F, intermediate cells lying against the old cuticle, and the persistent e dermis laying down fresh cuticle; G, 'brown' gland.

In *Scheloribates laevigatus* the histological changes that take place in integument have been observed particularly during metamorphosis to the ad stage, and in the harvest-mite *Trombicula autumnalis*, during the develo ment of the fully engorged larva to the newly emerged nymph. Individu were fixed in hot Bouin and stained with Mayer's haemalum and eosin, early, mid, and late periods of growth and developmental phases.

The epicuticle and endocuticle of the tritonymph of *Scheloribates laeviga* on emergence are deeply folded, the exocuticle is tanned, and the relativ thick, colourless endocuticle is clearly lamellated. The epidermis is thicker and its nuclei are distinct and evenly spaced. The cytoplasm contains basip granules giving the general epidermis an appearance in keeping with tinued function of secreting new layers of endocuticle after emergence . 5, A).

As the mite grows, the integument distends to accommodate the increased k. Midway in the growing phase the folds have been almost flattened out. e exocuticular layer is completely tanned and loses its former distinctly ellated appearance. The epidermis is reduced to about half its former pth when this mid-growth period is reached. The nuclei are still distinct d evenly spaced, but there is a noticeable reduction of the chromatin nules in the cytoplasm (fig. 5, B).

There is little change in the integument from the mid to late period of owth. The epidermis becomes attenuated and vacuolated and the endocuticle ly extended (fig. 5, C).

The prelude to the pre-adult developmental phase is marked by a thicken-g of the epidermis and accompanying mitosis and chromatolysis. Great mbers of nuclei are produced, far in excess of those needed, and there is accompanying profusion of deeply staining chromatin granules (fig. D).

During the early period of a developmental phase the epidermis differen-tes into two distinguishable regions—an inner one, containing most of the clei, in all stages of mitosis and chromatolysis, and the chromatin granules; d an outer one next to the endocuticle, distinguished from the inner one its clear acidophil cytoplasm and the absence of basiphil granules. The ter region further differentiates into a layer of flattened cells, each with a gle nucleus and non-granular cytoplasm (fig. 5, E). These flattened cells, indle-shaped and attenuated in side view and rounded in surface view, parate from the underlying epidermal region. Whereas these cells adhere to e old endocuticle, the persistent epidermis retracts and leaves in its wake exuvial cavity (fig. 5, F).

The persistent epidermis, with equally spaced nuclei, thickens, and two stinct, superficial, epicuticular layers are secreted. These outer and inner yers of the epicuticle in sections are probably the wax and polyphenol yers respectively. The flattened cells, still adherent to the endocuticle, ange from spindle-shape to oval, and in side view become more attenuated. ne old endocuticle is generally dissolved away. It is significant that these lls, called by former observers the intermediate cells because of their esence in the intervening space between the old and new cuticle, can ten be observed with their boundary wall having slightly migrated into the docuticle (fig. 5, F).

During the late period of development, comparatively few of these cells rsist and lie against the exuvium. This consists, finally, of the exocuticle d the epicuticle and some unabsorbed moulting fluid adhering to the inner rface. Before the eventual degeneration of these intermediate cells, an in-nse vacuolization of their cytoplasmic components accompanies the dis-lution of the endocuticle. By the time the intermediate cells have virtually sappeared and when the dissolution of the endocuticle has been completed, the epidermis has already laid down the potential adult exocuticle. It amber-coloured and distinctly lamellated before emergence.

On emergence, the exuvium is shed in a dry state; it is therefore reasona to suppose that, as in insects (Wigglesworth, 1933), the exuvial fluid is absorbed by the mite. The epidermis, at least in some regions, begins secrete the endocuticle before emergence, but the remaining bulk of this p of the cuticle is secreted afterwards.

In a harvest-mite, the epidermis of the unfed larva is very attenuated a the epicuticle is minutely folded. The distension of the integument of engorged larva results in a flattening of the endocuticle.

The ensuing process of changes in the integument follows on the lines that described for *S. laevigatus* except that only the epicuticle resists c solution and remains as the larval exuvium. When the developing m assumes the shape of the nymph, an epidermal splitting takes place and intermediate cells separate with the shed cuticle (the provisional cutic This provisional cuticle, which persists, is thin and the intermediate cells against it, in most regions, as a thick well-formed layer until just bef emergence (fig. 6, C). Histological evidence suggests that the intermedi layer of cells secretes enzymes in the moulting fluid which bring about granular disintegration of the provisional cuticle when the nymph emerg It is equally interesting that the active enzymes which are released, capable of dissolving the epicuticular larval exuvium (Jones, 1951).

The deposition of a provisional cuticle by the epidermis, the persisten of the intermediate cells as a layer until up to the time of emergence of the n stage, and the granular disintegration of this intermediate cuticle at the ti of emergence when the larval cuticle is shed in a dry state, are features whi distinguish the moulting process of a harvest-mite from that of tyroglyp and oribatid mites.

The changes that take place in the multiple glands are cyclical. Duri each growth phase these glands appear to attain a ripeness and compactn of structure which suggest it is the time when they are fully functional. Wh the epidermis differentiates and retracts, the ducts of these glands separ from the pores of the old plate and they are exposed to the active enzymes the moulting fluid which dissolves the endocuticle (fig. 5, F). If we accept view that living tissue is unaffected, it follows that the open ducts and expo cells have the capacity to resist the enzymes of the moulting fluid.

The glands deteriorate, the cytoplasm of most cells becomes increasin vacuolated, and the cytoplasmic components of some cells completely integrate as they drift into the haemocoel, where only the chromatin mate persists for any length of time. These multiple glands, although they their compactness, do not break down completely, since some cells appea persist and divide to form a new gland; the process being principally one rejuvenation. When the next stage emerges, these glands are complet formed.

The 'brown glands', like the multiple glands, retain their identity dur developmental phases. As the epidermis retracts, the duct of the gland aks away from the tubercle. The cuticle lining the lumen of the gland is d and the general deterioration of the cells is accompanied by mitosis and omatolysis; a golden brown exudate is ejected into the haemocoel. The erior dermal glands also undergo a process of rejuvenation and, like the er dermal glands, they are completely reformed by the time the mite is dy to emerge. The function of these glands is very obscure. Similar glands beetles are thought to be aphrodisiac (Wigglesworth, 1948), but since these nds, when present in mites, appear to be equally functional in all the juve- e stages, it is doubtful if they perform the function ascribed to the glands beetles. It is more reasonable to suggest, in the case of mites, that the udate of the multiple glands is an attractant, for promoting aggregation thin the community, the function being thus comparable with that of the ent glands of social insects.

The Relation of Cuticle Deposition to Muscle Development

The almost complete loss of segmentation in mites has resulted in the uscles being distributed as simple systems. However, there is a considerable velopment of dorso-ventrally placed integumentary muscles. Other tegumentary muscles, each attached at two sites upon the cuticle, extend in antero-posterior direction. These are quite well developed, but scattered their distribution.

A need for describing the different systems of integumentary muscles does t arise in this paper. The changes in the muscle systems illustrated in fig. 6 ve also been thought sufficient to clarify the intelligibility of the views ex- essed because the process of differentiation was apparently similar in all the tegumentary muscles.

In functional muscle the sarcoplasm stains pink and the chromatin of the rge oval nuclei stains dark blue with Mayer's haemalum and eosin. Its con- xion with the cuticle is usually obtained through intermediary processes own as fibrillae. These are derived from the epidermis (fig. 6, A and B). endons are also present in mites.

When muscles obtain effective attachment to the cuticle their transverse riations show up clearly. Their disappearance in old muscles is a sign that e connexion with the cuticle has been severed. Thereafter the old muscles fferentiate.

The muscles of the larval stage of a harvest-mite do not sever their con- xions with the cuticle or begin to disintegrate until about 6 to 12 days after omplete engorgement (fig. 7). The leg muscles became reduced to clumps of yoblast tissue at the bases. The degenerate integumentary muscles retained eir shape with regions of myoblast tissue connecting them up to the epi- ermis (fig. 6, B).

When the mite becomes immobilized owing to the severing of the muscles om the cuticle, it enters the phase of development of the next stage. In the harvest-mite this state of immobilization presaged the beginning of the nym phal stage.

The initial period of development was marked by regrowth (fig. 2). Th increase in size was accompanied by a change of shape. The mite becam elongated. This was followed by the epidermis moulding itself into the sha of the nymph (fig. 7). The mould, having the same spatial relations as t future nymph, now operated as a guide for the developing muscles. Hint (1949) reviewed theories on this problem in insect metamorphosis.

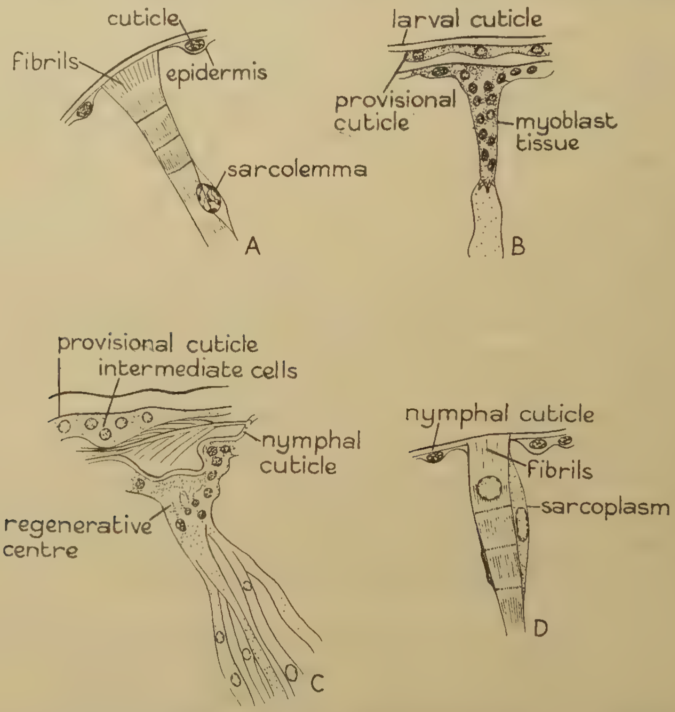


FIG. 6. Changes in skeletal muscle during the moults accompanying an atypical developmen phase in a harvest-mite. A, striped larval muscle attached to the larval cuticle; B, active regio of myoblast tissue intimately connected with the epidermis; C, regeneration of muscle tissu nearing completion; D, striped nymphal muscle attached to nymphal cuticle.

The unfolding muscles are destined to become attached to the integumen at predetermined points. They must, of necessity, be regenerated from th centres of myoblast tissue (fig. 6, B and C) and bridge the distances betwee these predetermined points. Now it so happened in the harvest-mite, tha these distances increased considerably during the early part of the phase c development, when new muscle tissue had already begun to form.

FIG. 7 (plate). Changes in form and the expression of the pupal state at each phase of de velopment in the growth of a harvest-mite. All figures are at the same magnification. Th larval stage shown in A is 0·25 mm. long. A, unfed larva. B, larva extended after engorgemen C, nymphal 'pupation'. D, nymph. E, adult 'pupation'. F, adult.

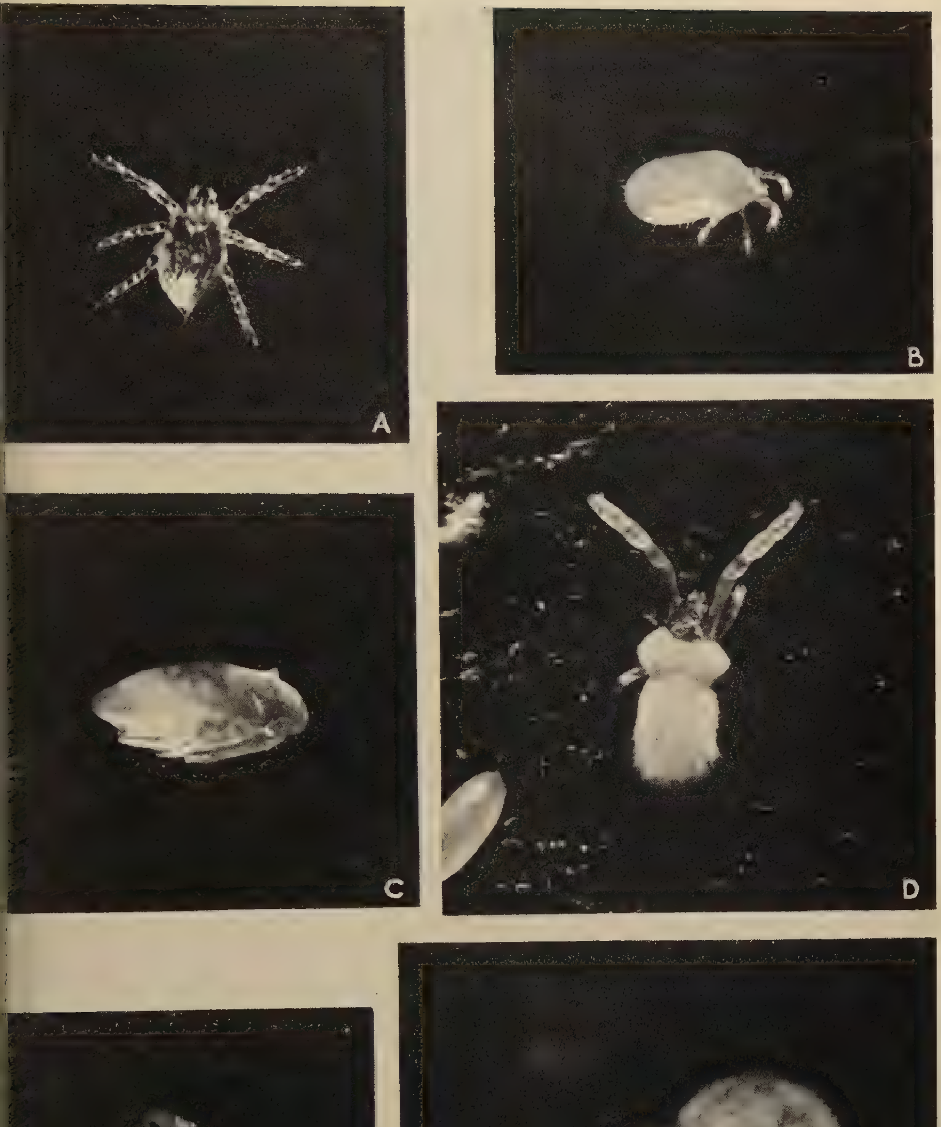


Fig. 7

B. M. JONES

When the second moult occurred, the proliferation of new tissues by the
erent regenerative centres was still in progress. By this time the different
tems, for example the gut and the various glands, were composed entirely
new cells. But despite their having obtained their proper form and dimen-
ns, differentiation of the various systems had not yet been completed. The
scles were unstriped and the myoblast centres were still active (fig. 6, c).
The epidermis on shedding the first-laid cuticle, redeposited fresh cuticle
h which the muscles gained effective attachment (fig. 6, d). It therefore
med reasonable to assume that the moult was aimed towards the achieve-
nt of this attachment of the muscles to the cuticular skeleton. It was also
sonable to suppose that since the other internal systems, at the time of the
ult, were progressing towards the final phase of their development, they would
t appear to need or to gain specifically from the induction of this extra moult.
Towards the end of the period of regrowth the epidermis fashioned a
iding mould for the developing internal systems. The fashioning of the
uld would therefore appear to be postponed until this process of regrowth
d been completed. It is also legitimate to assume, in the case of the harvest-
te, that this first mould was imperfect, because the pliability of the integu-
nt could have been restricted by the presence of cuticle initially deposited
n after the beginning of the moult.

The important point that calls for comment is that the developing muscles
ust perforce extend over distances which were substantially increased
ring the period of regrowth. This task, laid upon the muscles, probably
ts their development out of relation to the redeposition of cuticle. The
plication is, that the regenerative centres must continue to proliferate tissue,
yond the time when the first-laid cuticle would have reached the threshold
r allowing the attachment of the muscles. This conception would certainly
pear to be valid on the basis of the muscle tissue being still proliferated while
e moult of provisional cuticle occurred (fig. 6, c).

When development is typical, the formation of the muscle-ends coincides
th the development of the fibrillae, which in turn become integrated with
e first-laid cuticle. Only the one moult is therefore necessary. However, in
e harvest-mite the development of the muscles takes relatively longer,
hich puts it out of relation to the rate of deposition of cuticle. To counteract
is disharmony between the systems the first-laid cuticle was shed. There-
ter the rate of deposition of fresh cuticle was attuned to the final steps in the
velopment of the muscles and the fibrillae.

During metamorphosis the same sequence of events takes place. The nymph
sumes an elliptical shape on engorgement and growth takes place once again
hen the adult is first developing (fig. 7). In fact the same set of conditions
at induce an extra moult of provisional cuticle were present during meta-
orphosis.

A provisional cuticle is also formed during embryogenesis. It is similar and
rresponds to the provisional cuticles of the post-larval stages. The occur-
nce of this embryonic provisional cuticle suggests that pupa-formation is

expressed within the egg. It is noteworthy that the embryo of a harvest-m
swells considerably and breaks through the egg. However, the embryo
vested with its provisional cuticle remains in place (Michener, 1946). T
period of growth would appear to correspond to the period of regrowth whi
takes place during the early part of a post-larval phase of development. T
set of conditions which appear to induce pupa-formation in later developme
are therefore apparent during embryogenesis.

Discussion

In the light of the observations described in this paper, it seems reasonab
to suppose that the moulting process in mites is unique. The production
a provisional cuticle in some mites would also appear to be the critical fact
for distinguishing pupa-formation from a typical phase of development.

The provisional cuticle and the intermediate cells in the harvest-mite a
homologous with the 'apoderma' and the isolated cells respectively, noted
Trombidium fuliginosum by Henking (1882), and the 'Zwischenhaut' a
'haemamoebae' of *Atax bonzi* observed by Claparède (1868). But hitherto t
significance of the cells was not understood. Moreover, indefinite speculati
on their origin and function only led to confusion.

In the decapod Crustacea, Yonge (1932) noticed nuclei in the exuvial cavi
during the shedding of the old cuticle of the oesophagus of *Homarus*. But
was unable either to distinguish associated cytoplasmic components, or
discover the origin of the nuclei. He suggested, however, that they appear
to play a part in softening the 'chitin' of the old cuticle. The moulting proce
in mites probably explains the origin and function of the nuclei detected in t
exuvial cavity of *Homarus*.

Browning (1942) in his work on the spider *Tegenaria atrica* came to t
conclusion that the cells found in the exuvial cavity were discarded 'gran
locytes' which had functioned at the previous moult in laying down the ne
'exocuticula'. The figures of Browning are very definite, but it seems unusu
that a discarded cell should have to maintain itself from one moult to t
next in the epidermis before migrating into the exuvial cavity to under
disintegration. These 'granulocytes' in the spider bear a striking resemblan
to the moulting cells described in this paper. It is therefore tempting to su
gest that they may have a similar origin and function.

In view of the observations on the process of moulting in mites it wou
appear that the intermediate cells are capable of excavating the cuticle. Th
also become vacuolated and enlarged, thus assuming the appearance of sec
tory cells. It is therefore not amiss to suggest that they secrete the chitin
and protease enzymes responsible for the dissolution of cuticle.

So far, I have failed to locate, either histologically or chemically, derm
glands equivalent to those which, in insects, are both the possible source
the enzymes and the definite source of the 'cement layer' of the epicuti
(Wigglesworth, 1948). Since a cement layer is not present in the epicuticle
the mites studied in this work, the absence of such glands is understandab

The reasons for the unique moulting process in mites are not easy to fathom. e clean separation of the cuticle from the epidermis would appear to be a pler operation. In view of the exuvial cavity in mites being relatively very cious (fig. 3), it is tempting to suggest that in these small animals the dis- ution of the sometimes extremely hard cuticle is made more certain by the ulting cells being placed against the surface of the cuticle. The effusion of zymes from these cells would ensure greatest activation at the exposed sur- e of the cuticle.

The reasons for the production of a provisional cuticle during a phase of velopment in some mites have been tentatively put forward in the last sec- n of this paper.

Atypical development or pupa-formation in mites implies that the mite ring its development becomes enclosed by a provisional cuticle, having re or less the spatial relations of the final mould of the stage concerned. rip the mite of its provisional cuticle and pupa-formation ceases to exist. would also be legitimate to translate this proposition in terms of holo- tabolous insects. Poyarkoff (1914) postulated that only the developing uscles would appear to be in need of the extra moult which produced a sur- unding provisional cuticle. In mayflies, for example, the provisional cuticle e cuticle of the sub-imago) is shed in order that a few not quite fully rmed thoracic muscles only may gain proper attachment to the cuticular oskeleton. Pupa-formation in these insects at least is therefore even more rect than it is in the harvest-mite.

The production of an embryonic provisional cuticle is not uncommon in thropods. It is probably universal among insects (Wigglesworth, 1950). me spiders, and the nauplii of certain Crustacea on emergence from the egg, e, like a harvest-mite, invested with a provisional cuticle. Whether or not is condition during embryogenesis can be regarded as a form of pupation open to question.

It is a pleasure to thank Prof. Michael Swann for some valuable suggestions ade while the manuscript was being prepared. Dr. Wallace photographed e adult harvest-mite and he also very kindly enlarged my photomicrographs the other stages.

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Properties of the Minerals in the Exuvia of Crustacea

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With one plate (fig. 1)

SUMMARY

An unusual reaction between material in the exuviae of Crustacea and pure cold ercuric chloride, resulting in the deposition of a bright red compound, led to the iscovery that, contrary to expectation, the inner surface of the fresh moult, and even f the inter-moult exoskeleton, is strongly alkaline (pH = 9·1). This is near the ptimum for both alkaline phosphatase and carbonic anhydratase, which both may be ctive in resorption of mineral preparatory to ecdysis. Correlations between the dis-ibutions of the red Hg-compound, phosphate, carbonate, alkaline phosphatase, and igmentary pattern have been found in various Crustacea. The reaction with cold gCl_2 is given also by the millipede exoskeleton, the molluscan shell, the calciferous land-material of earthworms (pH 9·2–9·4) and by calcareous sponges, but not by chinoderm or vertebrate skeletons, and not by the exoskeleton of insects, spiders, and ntipedes, which lack mineral. The compound(s) with mercuric chloride are probably hiefly oxychlorides. A steady, if slight, solution of the skeletal carbonate and phosphate necessary to maintain the observed high pH, and to permit the formation of these ercury-compounds.

INTRODUCTION

WHEN fixing exuviae of *Asellus*, which are white and opaque, in cold concentrated mercuric chloride ($HgCl_2$) solution alone, a deposit of icroscopic bright red granules was formed on the inner surface. This triking colour-reaction is so unusual when using fixatives containing mercuric hloride that further investigation of its nature seemed justified. The reaction nd its products have been observed under the microscope and macro-copically, and the conditions determining the reaction investigated. Possible xplanations of the reaction were tested by the use of known reagents under omparable conditions, and it was eventually concluded that the reaction epended on an unusually strong alkalinity of the exuvia, interesting not only n itself, but also because it had seemed probable on previous evidence Richards, 1951, p. 223) that the moulting fluid of Crustacea would prove to e acid in reaction. The investigations and resulting conclusions are presented s far as possible in their logical sequence.

RESULTS

ncidence and properties of the reaction

The response was found to be given by the exuviae of other Crustacea—*aphnia*, *Chirocephalus*, *Gammarus*, *Porcellio*, *Potamobius* (*Astacus*), and *arcinides* (*Carcinus*), but not by those of insects, e.g. of naiads of the mayfly, f pupae of the gnat, and of nymphs of the stick-insect, or by those of spiders.

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This would seem to imply a correlation with the inorganic material, abund in the exuviae of Crustacea, but absent from those of the other two clas The exoskeletons of chilognath myriapods generally contain mineral mat and although exuviae have not yet been obtained, the exoskeleton of fres killed *Polydesmus coriaceus* gave an intense reaction. This was true also of fresh exoskeleton of Crustacea, especially when approaching ecdysis.

The red deposit was partly superficial, on the inner surface of exuviae, on the broken edges of fragments, and also within the exoskeleton. Sup ficial deposits indicate some solution of reagents before reaction, or of products of reaction before the final deposition, and in fact there was depo tion also on the surface of the medium and on any roughness of the glassw but variations in density of deposition on different parts of the exoskele (fig. 1) no doubt indicated local differences in concentration of react material. Deposition on broken edges and within the exoskeleton usu began in the 'pigment' layer (Yonge, 1932), i.e. the outer chitinous layer co posed of thin lamellae, and near its junction with the calcareous layer, which deposition later extended, in the inter-moult exoskeleton, though always in exuviae. The outer parts of the pigment layer also usually gav good reaction. The initial reaction, especially in fresh moults, gave a v diffuse, superficial deposit of fine red granules, showing a pattern (fig. related to the spines and other features of the moult of *Potamobius*, and follo ing the pigmentary pattern (Needham, 1942) in *Asellus*, and also to so extent in *Carcinides*. The colour was at first light orange, and darkened p gressively as the granules enlarged by the deposition of more material. W older, i.e. stored, moults, the larger granules were relatively more numer and a white deposit in the medium indicated more extensive solution material from the moult. The granules were spherical or subspherical w all shades of colour from orange, through vermilion, crimson, and purple almost black; but, in addition, more obviously crystalline black granu appeared. Even these were red when sufficiently thin to transmit light. T granules of the diffuse deposit frequently fused into irregular masses (fig. Both red and black granules were soluble in moderately strong mineral aci passing through a yellow intermediary compound to colourless 'ghosts', so of the material going into solution. The ghosts showed a concentric and rad construction. The yellow intermediary was not invariable and proba depended on the concentration of acid used. The red compound was sta when stored dry or in alcohol but faded in such acid mounting media balsam, and in the mother liquor. Alkalis turned the granules yellow and th sepia, without dissolving them. Concentrated $(NH_4)_2SO_4$ decolorized th rapidly and concentrated NaCl slowly.

FIG. 1 (plate). Photomicrographs of inner surface of exuviae of *Potamobius*, showing form and pattern of red granules formed by immersion in $HgCl_2$ solution. Granules are deposited in the region of spines of the exoskeleton. In places on the upper figure the out of colourless calcite spherules may be seen. The surface is curved, and out of focus p pherally.

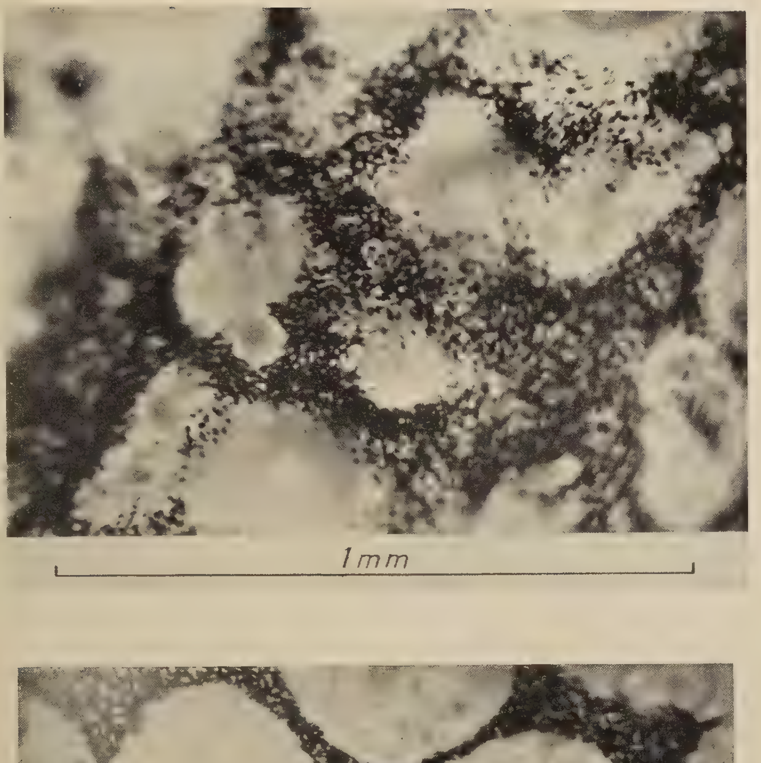

Fig. 1

A. E. NEEDHAM

Both red and black granules were birefringent. The former, when rotated tween crossed nicols, showed no variation in transmission, but the black ystals showed the typical extinction in two positions, at right angles to each her. The red granules therefore were probably masses of disorderly crystals, e latter single crystals.

entity of the red compound

The two main inorganic anions of crustacean and other skeletons are rbonate and phosphate, the latter in the form of a basic salt (p. 186), with lcium as the main cation of both. Both anions in suitable soluble form, e.g. $aHCO_3$, Na_2CO_3, and Na_2HPO_4, gave red compounds with $HgCl_2$ in 'test-be' experiments (often carried out on watch-glasses or slides for micro-opical observation, for comparison with the reactions of the biological aterial). All three reactions gave a red compound very closely resembling at formed in exuviae—in colour sequence, form of granules and properties ready described. The composition of the various mercury-compounds rmed with carbonates and phosphates is still uncertain (Thorpe, 1946, l. 7, p. 577) and varies with pH, concentrations of reagents, &c., and appears range from virtually pure carbonate (and perhaps phosphate) of mercury acid media through basic forms of these salts, with increase in pH, to ychlorides, $n(HgCl_2).m(HgO)$ (Mellor, 1923, vol. 4, pp. 829, 841, 1003; regory and Burr, 1926, p. 293), which contain neither CO_3 nor PO_4. The ose resemblance between the products obtained in all three of the reactions ove strongly implies that the same compounds, and therefore necessarily the ychlorides, were the main constituents of all, and likewise of the exuvial sponse. The most common oxychloride is $HgCl_2.2HgO$, which does in fact ist in red and black forms; further, the red form is known to turn purple, olet, or brown under the continued action of the mother liquors (Mellor, 23), just as in the exuvial response. The minerals mosesite and kleinite, hich approximate to the 1:3 salt, $HgCl_2.3HgO$, are yellow and red, and are refringent. Mosesite is often found (cf. p. 189) on calcite (Mellor, 1923).

Identification of the exuvial compound with oxychloride of mercury was pported also by (i) the eventual solution of the granules by the mother quor ($HgCl_2$); (ii) their destruction (p. 184) by NaCl and NH_4 salts; (iii) the eration of little CO_2 from them by acids; and (iv) the turning black of the anules on moderate heating, and the return to red on cooling; this is aracteristic of HgO and rules out the further possibility of the sulphide, nnabar, which also can exist in red and black forms but with the reverse olour-changes on heating. Sulphur is present in crustacean exoskeletons Richards, 1951), but no reaction for inorganic sulphide was obtained on test-g with a soluble lead salt.

Since both CO_3 and PO_4 tend to precipitate the same oxychlorides of ercury it may prove difficult to distinguish their respective shares in e observed responses (p. 188). Some contamination, at least, with the recipitating anion is to be expected. Washed granules gave a positive reaction

for phosphate with the rather sensitive reagent, 1-amino-2-naphtho
sulphonic acid, $NH_2.C_{10}H_5(OH).SO_3H$ (Hawk and others, 1947, p. 579).
equally sensitive test for carbonate was not available, but acid caused sc
evolution of gas from washed granules. Although the shell of *Helix aspe*
which contains much carbonate and little phosphate, gave a relatively s
reaction, in the test-tube carbonate was more reactive than phosphate, a
moreover, vertebrate bone, with a high ratio of phosphate to carbonate, g
no reaction. On the other hand, calcite crystals were abundant in exuviae,
indeed inside the inter-moult exoskeleton (Reid, 1943), but were very
reactive to $HgCl_2$ (fig. 1). Both PO_4 and CO_3 appear to have active and inac
forms (p. 186).

Carbonates form a red basic mercuric carbonate, $HgCO_3.3HgO$, wh
could theoretically constitute a considerable proportion of the observed co
pound without being evident among the oxychloride, but is probably p
cluded at the observed pH (p. 187). Another theoretically possible contamin
is mercuric pyrophosphate, $Hg_2P_2O_7$; this red compound is soluble in NaC
however, whereas (p. 184) the granules obtained were turned yellow and th
brown by NaOH. Nevertheless, the reaction of PO_4 *in situ* with soluble
and Co salts was that of pyro- rather than of orthophosphate. Other possi
red Hg-compounds, not due to CO_3 or PO_4, include the iodide which, ho
ever, is brighter in colour: the presence of iodide in such concentratior
highly improbable, particularly in the fresh-water forms.

Conditions necessary for the production of the red compound

While the general nature of the red compound seems reasonably cert
and its production in the test-tube is well known, its novelty as a result
reaction with biological material presents an interesting problem. Blood
other tissues which do contain soluble alkali carbonates and phosphates g
no reaction with $HgCl_2$, whereas skeletons contain only the sparingly solu
calcium salts. However, it was found that powdered $CaCO_3$ (Analar) gav
good response to $HgCl_2$ solution, with the deposition and growth of granu
on the surface of the powder, their form and colour resembling those
exuviae. $Ca_3(PO_4)$—formed by the action of Na_2HPO_4 on $CaCl_2$ in the p
sence of NH_4OH—gave no colour-response, but on adding solid Ca(OH
which alone gave an ochre compound with $HgCl_2$, the latter was replaced
a compound having essentially the red colour of the exuvial compou
$Ca(OH)_2$ showed the same behaviour with the secondary phosphate, CaHP
which also alone gave no coloured compound, and it seems possible theref
that phosphate is actually included in the red compound formed in
presence. The basic phosphate of vertebrate (Armstrong, 1952) and
crustacean skeleton is probably $Ca(OH)_2.3Ca_3(PO_4)_2$, hydroxyapatite, wh
therefore well might form this red compound under suitable conditions. H
solubility clearly is not essential.

More important is the correct pH. The red compound is very unstabl
acids and this no doubt explains its absence from material fixed in the u

rcuric fixatives, which generally include acids. $HgCl_2$ solution itself has a v pH and does in fact slowly destroy the red granules. Even normal tissues, about pH 7·0, do not give the reaction. It seems clear that alkaline conditions ust exist locally, and indeed the inner surface of fresh or recently stored uviae was found to have the surprisingly high pH of 9·0–9·1. Distilled water which moults had been steeped acquired this pH in 10–30 minutes, which monstrates further the definite, if slight, solubility of alkali necessary for e reaction with $HgCl_2$. The amount of CO_3 and PO_4 dissolved out by dis- led water was detectable qualitatively only after prolonged boiling. When eces of damped exuvia, to the inner face of which pieces of indicator-paper d been attached, were immersed in $HgCl_2$ solution, the pH of the papers mained near 9·0 for some time and only very slowly fell. There can be little ubt, therefore, that at the surface itself an alkaline pH may be preserved r a long time, aided by the slow solution of the mineral.

gnificance of the high pH of crustacean exuviae

The alkaline pH of the exuviae was given also by the outer surface of nerging *Carcinides*, and it seems highly probable therefore that it is the pH the moulting fluid. This is particularly interesting since the mobilization CO_3 and PO_4 from the exoskeleton before moulting (Robertson, 1937) had d, rather naturally, to the speculation that moulting fluid in Crustacea would rove to be acid in reaction (Richards, 1951, p. 223). It is no doubt significant at in insects, lacking mineral in their exoskeleton (p. 184), the moulting fluid neutral in reaction (Richards, 1951, p. 223); the alkaline pH would seem to e related specifically to the presence of mineral. Mobilization of the mineral pH 9·0 could scarcely be appreciable without enzyme action, but, on the her hand, it is improbable that the pH could merely drift so far after obilization at an acid pH. The proximity of pH 9·0 to the optimal pH (9·4) r alkaline phosphatase may be fortuitous, but it is interesting that Kugler nd Birkner (1948) found, at ecdysis, considerable changes in alkaline phos- hatase activity in the tissues of the crayfish. Moreover, the enzyme was con- entrated in the outer layers of the chitinous part of the exoskeleton, i.e. in e pigment layer (p. 184). A role in mobilization of PO_4 was one suggestion y the authors, since the activity in the exoskeleton was maximal just before edysis, but at that time there seemed little further support for this role in ew of the alkaline pH-optimum of the enzyme. This enzyme appears to be nerally associated with the formation of mineral skeletons (Armstrong, 952; Hanson, 1948) and a function also in the resorption of such material eems probable. It is now known that phosphatase is very versatile in its hosphate-transfer behaviour (Danielli, 1951) so that the postulated activity resorption of PO_4 is not highly improbable.

At the observed pH the mobilization not only of PO_4 but also of CO_3 must e enzymic, and it is significant that this pH is in fact near the optimum Roughton and Booth, 1946) for the activity of carbonic anhydratase (= anhyd- ase: Hoffman-Ostenhof, 1953) which has already been advocated (Richards,

1951, pp. 79, 106) as an agent in CO_3-mobilization. Although there is
evidence, up to the present, that this enzyme is more concentrated in
skeleton of Crustacea than in the tissues, it may prove to be the effect
agent. No other enzyme seems more probable from its mode of action, p
optimum, or distribution. Carbonic anhydratase controls the balance betw
CO_2 and H_2CO_3, and the sequent equilibria between the latter and HC
and $CO_3^=$ may be non-enzymic. The observed pH is probably slightly m
acid than the optimum for alkaline phosphatase, and may be a comprom
between the requirements for the two enzymes. The pH of $CaCO_3$ is
(Hodgman, 1950, p. 1493), that is more acid than that of the correspond
phosphate, $Ca_3(PO_4)_2$ (Britton, 1927). If this view of the mobilization of
two anions is correct, it is not surprising that they are so similar (p. 185) a
interdependent in their activities. The form in which $CaCO_3$ is deposi
depends on the proportion of phosphate present (Richards, 1951, p. 104) a
a similar interdependence during resorption also is to be anticipated. Ho
ever, it seems probable that mobilization of CO_3 alone sometimes occurs,
in the deposition of calcite crystals under the crustacean skeleton (p. 1
Again the molluscan shell and the skeletons of some other groups cont
little PO_4.

In view of the high concentration of alkaline phosphatase in the pigm
layer of the exoskeleton of *Potamobius* (p. 184) it is interesting that the depo
tion of the red Hg-compound tended to follow the pigment-pattern of
integument (p. 184), and an association between pigment- and mine
metabolism is possibly implied. The visible pigment of the larger decap
is chiefly in the exoskeleton, that of *Asellus* entirely in the underlying tiss
(Needham, 1942, 1949); in the latter animal it was the superficial deposit
the inner surface of the exuvia which coincided with the pigment-patte
whereas in *Carcinides* it appeared to be the deposit within the material of
exoskeleton. In decapods there was the added complication of a reddenin
carotinoids by the denaturing action of $HgCl_2$, but this colour, and the abse
of deposited granules, permitted distinction between patches of high ca
tinoid-content and patches of Hg-compound. On the edges of fragments
exoskeleton the distribution of the deposit through the thickness of the p
ment layer varied locally. The pigments of *Asellus* are mainly melanins, th
of *Potamobius* mainly carotinoids and those of *Carcinides* rather numerous
is not yet clear which, if any, are specifically related to alkaline phospha
activity, or to the distribution of minerals in the exoskeleton. Kugler
Birkner (1948) were unable to localize PO_4 in the exoskeleton, and attem
to do so, by using the amino-naphthol-sulphonic (ANS) technique (p.
have not been successful, largely no doubt because it causes too m
mobilization of the phosphate. When sections of exoskeleton, fixed i
mixture including ammonium molybdate and H_2SO_4, were treated with A
all layers below the epicuticle showed a diffuse blue colour; and althoug
appeared somewhat denser in the pigment layer this was possibly due to
greater density of lamellae in that layer. On the edges of fragments of

eleton of *Carcinides*, treated directly with the ANS technique, there was a avier colour in the superficial part of the pigment layer than elsewhere. he colour-distribution showed the same local variations through the depth the layer as the Hg-compound (p. 188) and may be a genuine indication of rying PO_4-concentration in the material.

rther distribution of the Hg-reaction

In the search for other biological materials which might show the Hg- sponse, the products of the calciferous glands of the earthworm were found react positively, though not in all individuals. The concretions contain lcite and a little phosphate and the secretion of the glands has a pH (9·2– 4) very near that of the crustacean exuviae (Robertson, 1936). The reaction peared to be with cementing material and not with the calcite crystals emselves (cf. p. 186). In one individual the semi-solid contents of the glands so gave a good response, but here the colour was a duller brick-red, probably a carbonate. The ANS test was positive for PO_4 in the concretions. Al- ough few annelids other than the serpulids (Hanson, 1948) build calcareous eletons, it seems probable that their carbonate- and phosphate-metabolism fundamentally as in the related shell-building phyla, the Arthropoda and ollusca.

The macruran gastroliths may be expected to give a positive Hg-response, t only because of their role in ecdysis, but also because of the similarity of eir anatomical relationships to those of the earthworm calciferous glands. he digestive diverticula of *Carcinides* and *Helix* contain concretions of osphate or carbonate (Robertson, 1941), but these tissues gave no colour- sponse to $HgCl_2$. A positive response conceivably might be obtained when e mineral is in process of mobilization, but the fresh liver-tissue of *Helix* oved negative after regenerating shell for 14 days.

Moribund, but not fresh, calcareous sponges of several genera gave a posi- e reaction, but the skeletons of *Asterias* and *Echinus* did not.

I am greatly indebted to Dr. O. L. Thomas, who first pointed out that the d Hg-compound was deposited from solution and was unrelated to the lcite spherules, to Dr. J. R. Baker, Professor M. M. Murray, and Mr. M. McG. Russell, who, at different times, examined the granules under the larizing microscope and discussed various related points, to Dr. J. W. nnett for advice on facts of chemistry and for reading the manuscript, to iss B. A. Evans and Mr. H. W. Appleton who independently measured the H of the moult, and to Mr. P. L. Small and Mr. M. Lyster who prepared the icrophotographs.

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Spicule Form in *Leucosolenia complicata*

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SUMMARY

The tri- and quadriradiates of *Leucosolenia complicata* are described in four aspects: rface view, transverse view, side view, and planar view. The spicule form and size ry with the distance from the oscular edge in all four cases.

In planar view (when the eye is directed along the optic axis) the spicule rays usually pear straight and thus exhibit a tendency to grow in planes which intersect in a line ving the direction of the optic axis. The angle between the oscular ray planes (the nar angle) is always greater than 120° and the spicules hence belong to the 'second- y sagittal' class of von Ebner. The planar angle is not closely correlated with the optic gle and varies from about 160° at the oscular edge to about 140° at the base of the be. This variation precludes the possibility that the angle is simply controlled by the ystallizing properties of calcite.

The curvature of the choanoderm is a factor controlling spicule form because the rmative calcoblasts move in contact with this layer when secreting their respective ys.

Reasons are given for returning *L. coriacea* (Montagu) to a separate genus, *Clathrina*.

INTRODUCTION

IN a previous communication the author (1954) described the orientation of the optic axes of spicules of *Leucosolenia complicata* (Minchin), and owed that both the optic angle and the form of the tri- and quadriradiates ry with the position of the spicule along the length of the oscular tube. owever, only the form as seen in surface view was then considered, and in is paper three other aspects of the spicules will be described and discussed relation to their position in the oscular tube. Altogether the four aspects e: (1) 'surface-view', for which the eye is directed at right angles to the basal y with the oscular rays symmetrically disposed on each side of the field;) 'side-view', when the line of vision is perpendicular to the plane containing e basal ray and optic axis; (3) 'transverse-view', when the spicule is seen, as it ere, in transverse section of the oscular tube, i.e. with the basal ray directed ong the line of sight; and (4) 'planar-view', when the optic axis coincides ith the direction of vision. In all four cases camera lucida drawings have been ade which represent the projections of the spicule into a given plane rpendicular to the line of vision, namely, the plane brought into focus by e microscope.

METHODS

The spicule form has been studied by using isolated spicules and thick sec- ons of the oscular tubes. Axial longitudinal sections were cut at a thickness 30 μ, as described in the previous paper (Jones, 1954), and similarly trans- rse sections at 125 μ thickness. The dimensions of the tubes used were easured before sectioning.

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The method for obtaining and mounting isolated spicules was the same described in the preceding paper. The quadriradiates were usually picked by using their gastral ray tips as handles, because then all four aspects co be obtained without having to dislodge the spicule. Sometimes dislodgem had to be attempted, however, for a particular aspect, before the balsam h dried, but this should be avoided if possible as the spicules are very sm and springy. The triradiates were mounted by using the tip of one of th oscular rays, the needle lying in a plane parallel to that containing the ba ray and optic axis. It was necessary to tilt the supporting needle considera to bring the spicules into the correct orientation for one or other of the aspe and a modified camera lucida had to be employed to avoid changes magnification on racking the microscope up and down; the modification described in the previous paper.

Having set a spicule for side-view observation, the optic angle and len of the basal ray were directly measured and a calculation made of the len of the projection of this ray in a plane perpendicular to the optic axis. T spicule could then be set for planar view by tilting and rotating the support needle until the basal ray had this apparent length and the oscular rays w symmetrically arranged on each side of the field. It is not practicable to ori the optic axis directly, with crossed nicols, because the spicule in air gi anomalous optical effects owing to the convergence of the light by the r and to internal reflexions.

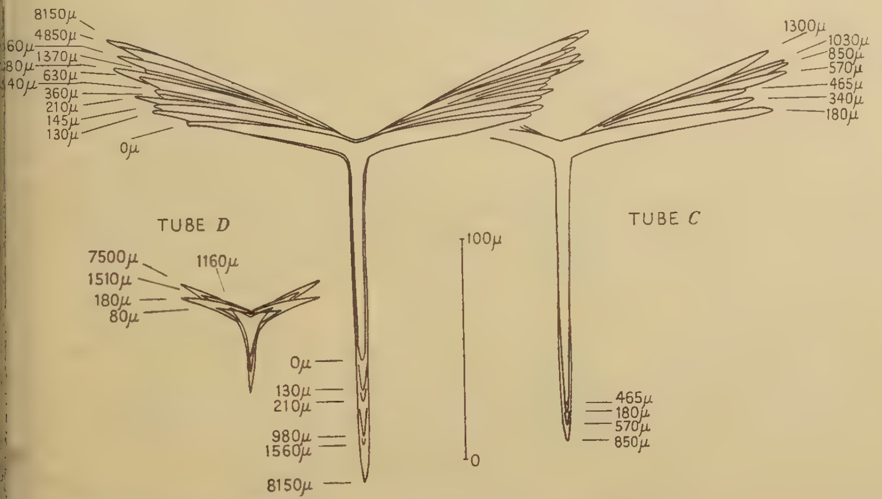
As before, the approximate position in their original tube of the isolat spicules was determined by comparing surface-view drawings with those ma previously of 'inner' tri- and quadriradiates already in position in pieces the wall.

RESULTS

Tri- and quadriradiates in surface view

In the previous paper (Jones, 1954) superimposed drawings were depict of inner tri- and quadriradiates of two tubes, *A* and *B*, derived from differe specimens, and the continuous variation in curvature of the oscular rays a the size of the spicules was obvious at a glance. In fig. 1 similar drawings a given for two other tubes, *C* and *D*, taken from the same specimen as tube tube *C* being a thin tube, 2–3 mm. long, of approximately $750\,\mu$ diameter in t middle, and *D* a wide tube, 9 mm. long, elliptical in cross-section, with lar and small diameters of 2 mm. and 1 mm. respectively. Both give essentia the same pattern as for the other two tubes, but the two patterns are n identical, so that the factors responsible for spicule form have not been dist buted in quite the same way in the two cases. Once again only 'inner' quad radiates were used; the 'outers' of tube *D* were found to correspond larg with the inners of the same level, but a few resembled inner spicules clo to the oscular edge, while others, occurring near this edge, had shapes simi to those slightly behind. These outer spicules were formed when the tu was younger and conditions of growth not the same as at the time of fixatio hus one would expect, since the tube grows in length over practically the hole surface (Jones, 1952), that some of the outer, older spicules would rrespond in shape to the more recently formed spicules sited nearer the cular edge.

The curvature of the oscular rays of triradiates resembles that of the quadri-diates occurring at the same level. In both spicule types it is rare to find act bilateral symmetry; almost invariably each oscular ray is slightly differ-t in length and curvature from its partner, and for this reason it is better to



G. 1. Superimposed surface-view drawings of inner tri- and quadriradiates of two oscular pes, *C* and *D*, derived from the same specimen of *L. complicata*. The oscular ray curvature d the size of the spicules vary with the distance of the spicule from the oscular edge, but e two patterns are not exactly similar. Superimposed drawings of young spicules of tube *D* are also depicted.

perimpose the drawings with the oscular rays symmetrically arranged about e mid-line, rather than to have the basal rays exactly coincident. Such an rangement avoids inconsistencies between the two sides of the composite awing.

Superimposed drawings of small spicules are also presented in fig. 1. It is ear that the founder cell which secretes each oscular ray sets out along a path culiar to the position of the spicule along the length of the tube, and that e thickener cell then alters the apparent direction of growth by depositing ore material in the angle between the oscular rays, as this angle tends to be naller than that of the fully grown inner spicules found at the same level.

Apart from the variation already noted, the proportions of the spicules vary nsiderably. Thus in surface view the basal ray may be slightly shorter than e oscular rays, though usually it is longer. However, the oscular rays are clined to the surface-view plane and hence appear shorter than they actually e, so that the variation may not be so evident when the actual lengths of the ys are compared. There is also some variation in the thickness of the rays from spicule to spicule, and mention must be made of the double-curvatu that is occasionally seen in the oscular rays (fig. 7, A; see p. 200) and has be described by Minchin (1904). This was not so common in the mater examined as might be expected from Minchin's description.

In transverse view

If the spicule is set so that its basal ray axis coincides with the line of sig the oscular rays are seen to diverge from the spicule centre and to be gen curved along their lengths, with their gastral surface on the concave si This is not always the case, however, for spicules with a slight doub curvature, or with a slight reverse curvature, have been observed. Wh examined in transverse sections of the tubes, the oscular rays of the inn spicules are found to follow closely the curvature of the choanoderm (exce ing distortion due to dehydration and sectioning, &c.), and there can be doubt that the founder cells move in contact with this cell layer when secreti the oscular rays. The variations in curvature are presumably largely due to t unevenness of the choanoderm, caused by the protruding gastral rays of t quadriradiates.

The outer spicules, on the other hand, particularly those at the base of t tube, commonly do not fit the curvature of the wall so well, and once ag the explanation lies in their earlier formation, when the diameter of the tu was presumably smaller. Fig. 2, C shows this well; the drawing was taken fr the base of a diverticulum formed by the outbulging and remoulding of t tube wall, and the original spicules, one of which (a triradiate) is shown, ha an orientation at right angles to that of the newly forming quadriradiat which have their basal rays pointing to the apex of the bulge, as in the no oscular tubes of *L. lieberkühnii* (Bidder, 1891; Minchin, 1908). The oscu rays of the triradiate consequently do not fit the wall very well, and in ti this spicule would probably have been shed when the pinacoderm had adjust itself to the new curvature. Poorly fitting spicules can be passed through t cell layer, as is observed, for example, when an oscular tube contracts und unfavourable conditions.

A comparison of small tri- and quadriradiates (fig. 2, D and E) usua reveals that the choanoderm is quite flat for the former but is bulged out by gastral ray rudiment of the latter. This outbulging suggests that the mesogl is firm, since the gastral ray must be pushing against some support, and expe mental evidence that this is the case has been obtained by the author (Jon 1952) and will be published in due course. The depression of the choanode by the young quadriradiates is presumably the cause of the kinks seen in th oscular and basal (see later) rays, since the calcite fills the hollow and ma the ray axes appear to bend into it. In the triradiates, on the other hand, oscular and basal ray axes run almost straight out from the spicule centre a then bend towards their pinacoderm side, before continuing along the ch noderm curvature (figs. 2, A, 4, D). This arrangement suggests that the spic ntre is raised from the choanoderm during the secretion of the proximal 30 μ so of the rays. Some of the quadriradiates likewise have a double curvature addition to the proximal kink on their oscular rays when seen in transverse ew (fig. 7, c).

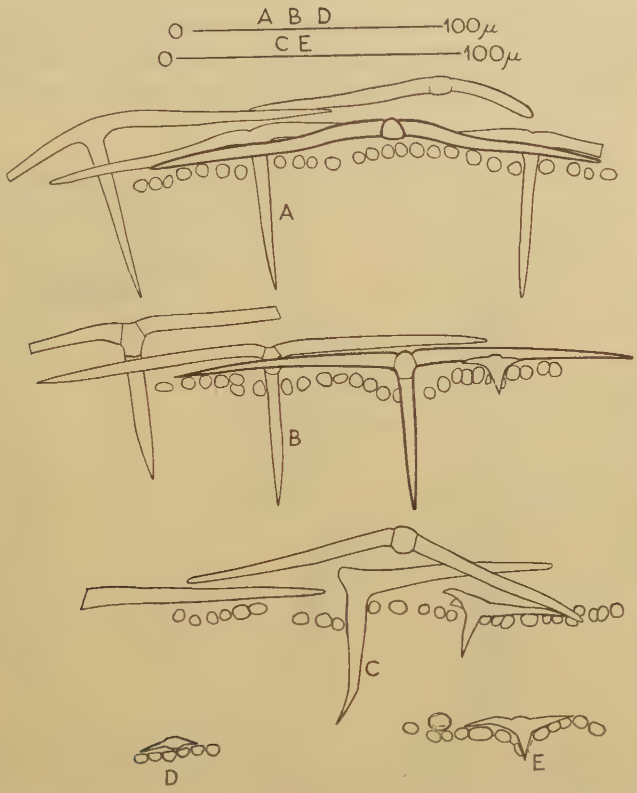


FIG. 2. Camera lucida drawings of spicules seen in transverse sections of an oscular tube of *L. complicata*. (A) A triradiate and some quadriradiates; (B) quadriradiates; (C) a poorly fitting triradiate at the base of a diverticulum; (D) young triradiate (obliquely oriented); E) young quadriradiate. The difference in curvature of the oscular rays of the triradiate and the quadriradiates is conspicuous. Note that the young quadriradiate (E) has bulged out the choanoderm.

With isolated spicules in transverse view the same picture is presented, although the angle of divergence of the rays shows considerable variation with wide tubes like tube *D*. This is partly explained by the elliptical cross-section of this tube and by the presence of outer spicules, but also to some extent by the fact that small errors in the setting of the spicule can appreciably alter the angle between the rays. However, for tube *C* the superimposed drawings of isolated spicules in transverse view form a consistent picture (fig. 3, B). As would be expected, the angle of divergence of the oscular rays tends to increase with increasing diameter towards the base of long tubes.

Side view

Side-view drawings of tri- and quadriradiates have already been giv (Jones, 1954), together with an account of the curvature of their basal ra (figs. 4 and 5), but no mention has yet been made of the shapes of the gast rays. For quadriradiates over most of the tube surface these have three po tions, a short proximal region roughly directed at right angles to the opt axis, then a longer central part directed perpendicularly to the inner surfa

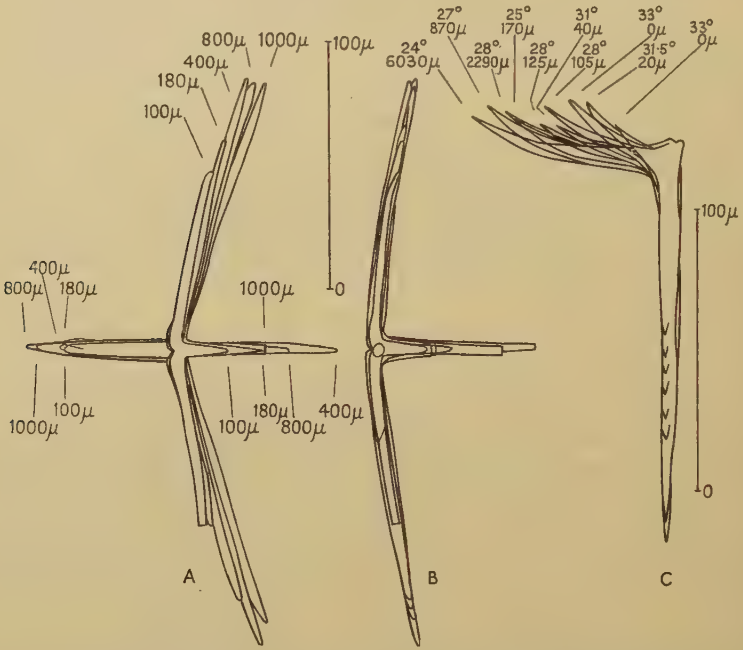
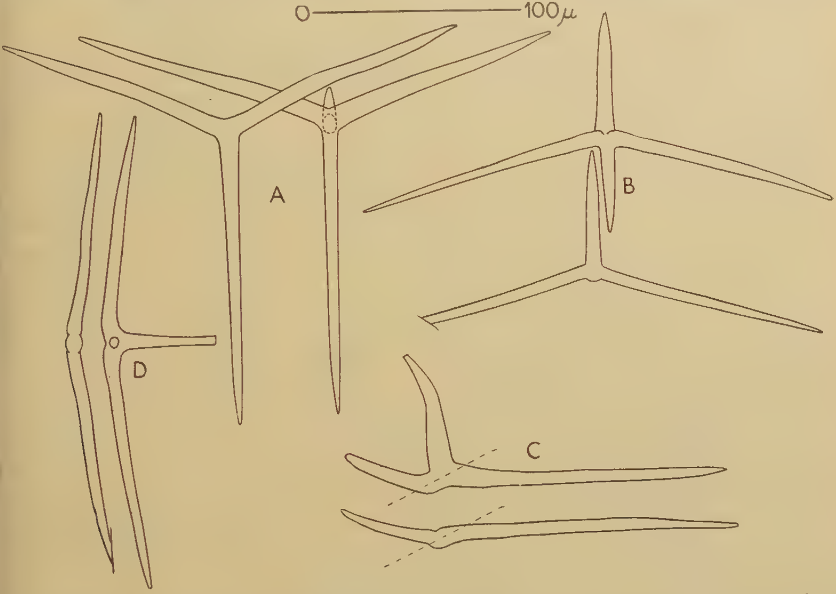


FIG. 3, A and B. Superimposed camera lucida drawings of isolated quadriradiates of tube as seen in (A) planar view and (B) transverse view. The planar angle decreases with increasi distance from the oscular edge. (C) superimposed camera lucida drawings of quadriradiates tube *A* seen in side view in axial longitudinal sections of the tube. The shape of the gastr ray varies continuously from one extreme to the other. The optic angles and distance from t edge are indicated.

of the tube, and, finally, a gradually curving distal portion which poin roughly towards the osculum. This distal region is always nearly perpendicul to the optic axis, but deflected slightly from the true perpendicular in t direction of the internal water current. However, at the oscular edge itself different type of gastral ray occurs (fig. 5, A and G). This lacks the straight ce tral portion and generally curves along its whole length into a direction f from perpendicular to the optic axis. In between these two extreme typ occur intermediate shapes (fig. 3, C), but there is no close correlation betwe the shape and the position or optic angle of these intermediates as the figur the drawing indicate. However, most of the variation in gastral ray curva-re is found within 150 μ from the oscular edge, i.e. the extent of the oscular m. A full discussion of the factors controlling the shapes of the gastral rays ill be given in a later paper.

It has been shown previously (Jones, 1954, and, for syconoid species, Wood-nd, 1905) that the length of the basal ray varies with the position of the icule along the length of the oscular tube. Fig. 6 is a graph showing the riation for tube *A*, and was obtained by measuring the lengths of the basal



IG. 4. Four aspects of a triradiate and a quadriradiate of tube *C* situated approximately ,000 μ from the oscular edge. (A) surface view, (B) planar view, (C) side view, (D) transverse iew. Note the difference in curvature of the basal rays in side view and of the oscular rays in transverse view.

ays on the sections used for the graph of the optic angle against position lready given (Jones, 1954), with the addition of a few readings taken directly rom the piece of wall left after the sectioning. Not all the young spicules een were measured, but there is no doubt that they occur throughout the ength of the tube. At each level young inner spicules may be found with engths varying from a few μ up to the maximum size occurring there, but the uter spicules only give a restricted range of size varying up to the maximum. These facts are consistent with the development of the spicules to their full xtent while in contact with the choanoderm, and their subsequent separation from this cell layer by the formation of new spicule rays in between, or by the ecretion of fresh mesogloeal jelly by the choanocytes (since some basal rays have separated evenly all along their lengths from the collar-cells without the presence of rays in between). The variation in size of the outer spicules represents a natural variation of the fully-grown condition.

The maximum size reached varies with the distance from the oscular edg
Over the first 250 μ it increases rapidly, but thereafter much more gradual

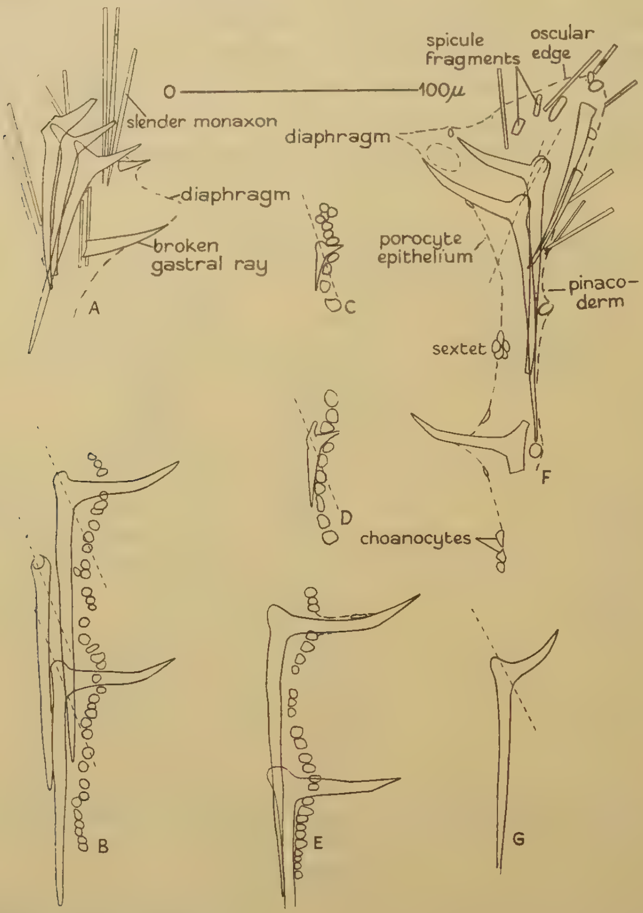
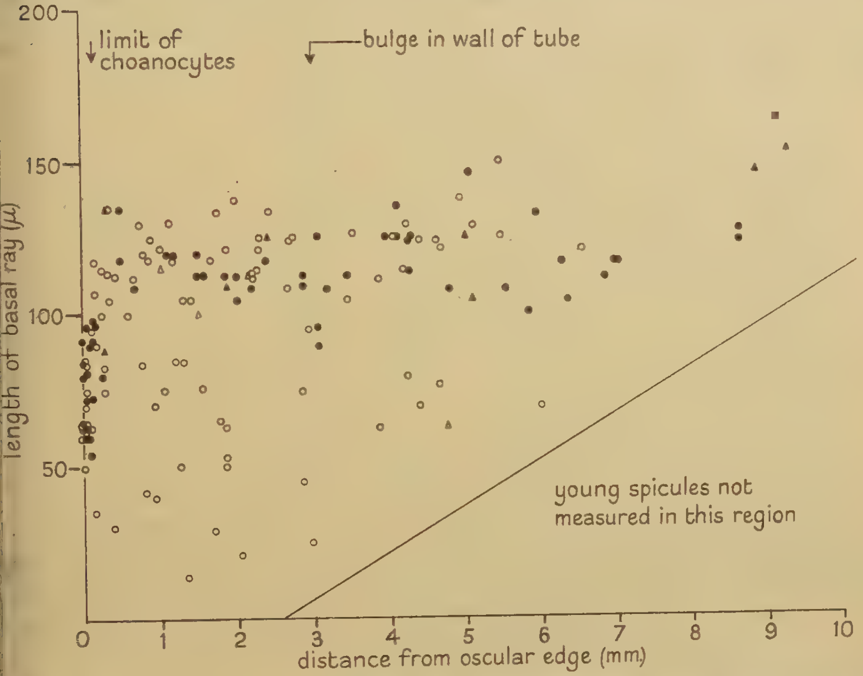


FIG. 5. Camera lucida drawings of tri- and quadriradiates seen in axial longitudir
sections of an oscular tube of *L. complicata*. (A) quadriradiates at the oscular edge; (B) tv
quadriradiates and a triradiate situated about 5,000 μ from the edge; (C) young quadriradia
(at about 2,000 μ); (D) young quadriradiate (at 1,000 μ); (E) quadriradiate with a very co
spicuous kink in its basal ray (at 2,000 μ); (F) quadriradiates just behind the oscular edg
(G) quadriradiate at the oscular edge. Note the variation in the shapes of the gastral ray
and the fit of the basal rays of the young spicules to the curvature of the choanoderm. T
direction of the optic axis is indicated by a broken line in some cases.

and similar results have been obtained with four other oscular tubes. In vie
of the corresponding variation in the optic angle with position, it might l
supposed that a long basal ray is correlated with a small optic angle and co
versely. However, the relationship is not a close one because (*a*) spicules wi
optic angles as low as those found near the base of the tube occur at tl
oscular edge, but no basal ray as long as can occur only 200 μ from the edge

nd at the edge itself; (*b*) the spicules of tube *A* are larger than those of be *B*, even though the optic angles of the former are on the average 3° eater; and (*c*) *L. variabilis*, which, if anything, has smaller optic angles for quadriradiates (only a few have been measured), characteristically has short sal rays of about 80 μ length. Thus the length of the basal ray does not de-



IG. 6. Graph showing the relation between the length of the basal ray and the distance from e oscular edge for outer and inner tri- and quadriradiates of tube *A*. Quadriradiates: inner, ○; outer, ●: triradiates: inner, △; outer, ▲.

end solely on the crystallization taking place more easily in certain directions elative to the optic axis. More probably it is controlled by the availability of etabolites, since short rays are found in the oscular rim where choanocytes re absent. It may also be significant that in the region of a bulge in tube *A*, he lengths of the basal rays of the outer spicules are slightly smaller than one ould expect (fig. 6). However, there may be a causal relationship between he optic angle and the length of the basal ray, which is masked by other actors.

n planar view

When the optic axis is coincident with the line of sight the oscular rays of uadriradiates usually appear quite straight (except for the extreme proximal ortions) and bound an angle that is always greater than 120° (figs. 4, B and , B). The four rays thus tend to lie in planes which intersect to form a line aving the direction of the optic axis, and because the angles between the planes taken in pairs are not equal, the spicules belong to the 'seconda sagittal' class of von Ebner (1887). In the triradiates the oscular rays genera curve gently when seen in planar view (figs. 4, B and 7, D), while some quad radiates also present a similar picture (fig. 7, C), so that it must be emphasiz that there is only a tendency for uniplanar growth of the rays to occur. Ho ever, the oscular rays are most nearly straight in planar view, while aberra spicules like that depicted in fig. 7, B show the planar growth well, so th there is good reason for maintaining the concept.

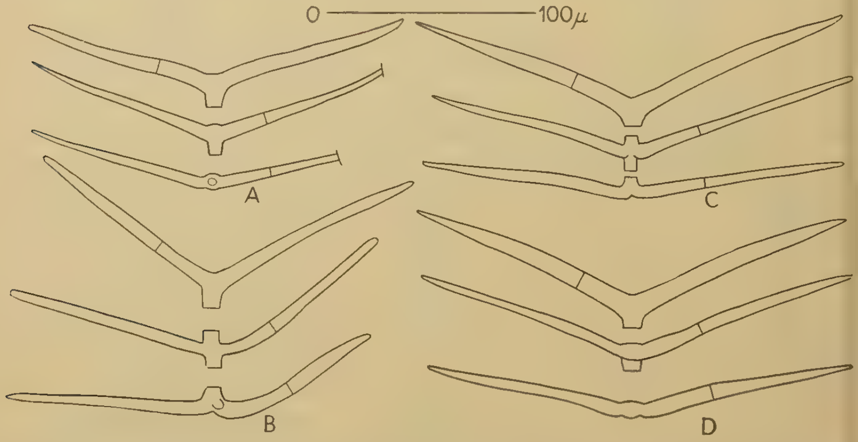


FIG. 7. Surface, planar, and transverse views (from above downwards) of (A) a quadriradia with oscular rays doubly curved in surface view; (B) an aberrant spicule with the oscul ray planes inclined at 123° to each other; (C) a quadriradiate with double curvature in tran verse view; and (D) a triradiate similar to (C).

When the planar views of isolated spicules are compared (fig. 3, A) the ang between the oscular rays (the 'planar angle') is found to vary, and furthe more to decrease with increasing distance from the oscular edge. This te dency is revealed by the figures given in table 1, derived from isolated spicule of tube *C*. Some inconsistencies occur, representing variation caused b different conditions of development, perhaps, or inaccuracies in the settin of the spicule for planar view (though it has been found that rotating the bas ray about the spicule centre in a vertical plane by as much as 7° on either sid of the orientation which sets the optic axis coincident with the microscop axis only alters the planar angle by one or two degrees). However, the overa tendency is clear; the planar angle is high at the oscular edge and decrease towards the base of the tube, the rate of change being greatest over the fir few hundred μ.

When the planar and optic angles of all the isolated spicules examined wer considered, no simple relationship between them became apparent, though rough non-linear correlation is given by the spicules of tube *C* (table 1 However, this is probably a coincidence and due to the tendency for bot angles to vary with the distance of the spicule from the oscular edge. In fig.

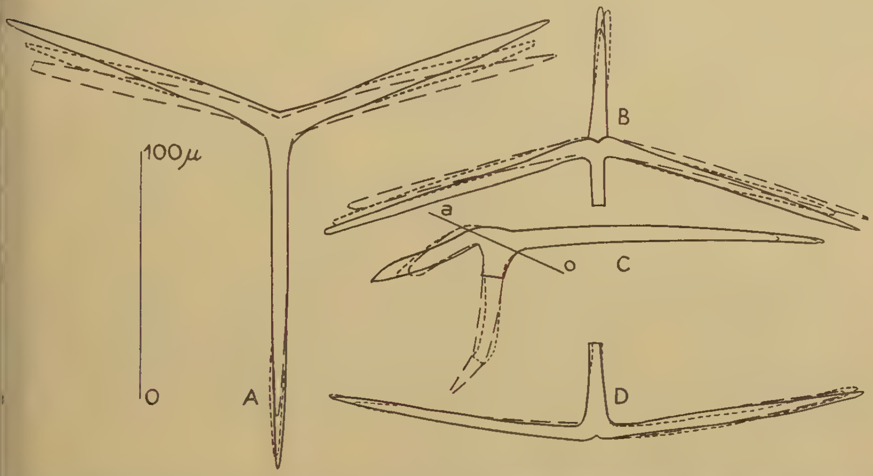
awings of three quadriradiates are superimposed to show that spicules with e same optic angle (25°) can have different planar angles.

TABLE I.
Isolated spicules of tube C

Tri- or quadriradiate	*Planar angle* (°)	*Optic angle* (°)	*Position* (μ)
Q	161	33·5	100
Q	159	33·5	100
Q	154	28·5	180
Q	152	27	450
Q	151	25	400
Q	149	25	500
Q	149	25	900
T	149	26	1,000
Q	148	26·5	800
Q	147	25	800
Q	145	26	1,200
Q	145	25	1,000
Q	145	25	1,200
T	144	23	900
T	140	27	400
T	140	23	1,300
T	139	25	1,200

The optic angle of the triradiates was measured against the gastral edge of the basal ray.

The oscular rays generally appear thicker when seen in surface view than hen in planar view, because the rays are elliptical in cross-section, the long



IG. 8. Superimposed drawings of three isolated quadriradiates derived from tube *C*, demon- trating that spicules with the same optic angle (25°) can have different planar angles. (A) surface view, (B) planar view, (C) side view, (D) transverse view.

xis of the ellipse coinciding with the direction of the optic axis. The thickness f the rays allows them to be shaped in surface view without there being a orresponding shaping appreciable in either planar or transverse views. Thus the oscular rays may appear slightly doubly-curved in surface view throu an uneven distribution of material by the thickener cell, and yet be straig in planar view and have gastral surfaces singly curved in transverse vie Usually, however, a double curvature in one aspect is revealed by a kink the ray in planar view (e.g. fig. 7, A), or by curvature in any or all of the oth aspects. In fig. 7, C and D the curvature of the oscular rays in transverse vi is also seen in planar view, and this gives the reason why uniplanar growth not so well defined in the triradiates, namely, because in transverse view t rays are usually doubly-curved, as described earlier.

The minimum planar angle measured was 139° and the maximum, 162°.

Monaxons

These have already been described by Minchin (1904) and the auth (1954).

DISCUSSION

An explanation of the tendency for planar growth and the planar angle w be deferred until some more evidence has been presented in a later pape For the present, however, it should be noted that the planar angle cannot accounted for directly by the process of crystallization, because equivale crystal faces must grow at the same rate when bathed by the same mediu and therefore the rays can only develop freely in directions such that the faces are symmetrically disposed at the ray tip. The planar angle for the t gonal calcite crystal would have to be 120°, therefore, but as observed abo it in fact varies continuously from 139° to 162°. Hence the planar angle cann be related to the crystallographic angles of the calcite crystal, particularly it is not closely correlated with the optic angle. Furthermore, the increa above 120° of the planar angle is not due to the tilting forward of the formati cell sextet (Jones, 1954) and the consequent squashing of the oscular r founder cells (which could conceivably splay out the precursor rodlet because the planar angle is greatest at the oscular edge, where the optic ang is greatest, and hence where there is least tilting.

In 1898 Bidder attempted to explain the surface-view appearance of t tri- and quadriradiates as the resultant of two growth factors, (*a*) the tenden for the rays to grow in planes intersecting symmetrically to form a line in t direction of the optic axis, with equal angles of 120° between the planes tak in pairs, and (*b*) the restricting influence of the bounding surfaces of t mesogloea. As the optic axis is inclined to the surface of the tube, the oscul planes cut the cylinder obliquely and each oscular ray, developing along t intersection of its plane with the surface, hence traces out part of an ellip when in surface view. However, as shown above, the planar angle is n 120°, so that Bidder's explanation requires modification, but none the less it still true, on geometrical grounds, that the path of the oscular ray founder c coincides with the intersection of the plane of growth and the surface of t choanoderm. Bidder's explanation fails only in that he postulates a crystal

phic factor to control the planar angle, which he assumed was 120°, as is
case with the perregular spicules of *L. coriacea* (Montagu).
The results of this and the preceding paper have shown that the skeletal
ganization of *L. complicata* is a complex affair. Besides the orientation of the
cules there is an orientation of their crystalline content, depending on the
tance from the oscular edge; and moreover there is a continuous variation
spicule form, as seen in the four aspects, along the length of the oscular
e. Clearly *L. complicata* is on a higher evolutionary plane than *L. coriacea*,
ich possesses only triradiately symmetrical spicules with optic axes always
arly perpendicular to the wall; and the advantage of the superior organiza-
n would seem to lie in the much better fit of the T-spicules to the oscular
ge, together with the retention of the Y-spicules, which are better adapted
the support of the basal regions of the tube (Bidder, 1898). The difference
the skeletal systems of the two species, together with the differences in their
erall growth processes (Bidder, 1891; Urban, 1906) and in the position of the
clei of their collar-cells (Robertson and Minchin, 1910), plus the presence
a sieve-like membrane across the osculum of *L. coriacea* (Minchin, 1892),
rely warrant the reversion of *L. coriacea* to a separate genus (*Clathrina*)
ce more (see *Plymouth marine fauna*, 2nd edition, 1931, p. 59).

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A Comparative Study of the Spermatophores of three Scyllarid Lobsters (*Parribacus antarcticus, Scyllarides squammosus,* and *Scyllarus martensii*)

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SUMMARY

1. In *Parribacus antarcticus* and *Scyllarus martensii* mitotic division of germinal ithelial cells produces primary spermatocytes, whereas in *Scyllarides squammosus* is function of the germinal epithelium is limited to comparatively few large, oocyte-e cells.

2. Metamorphosing spermatids in all three species are expelled from the sacculi the testis by the rhythmical activity of their germinal epithelium. This probably counts also for the clumping of the spermatozoa in the collecting tubule; each clump presents the extended contents of a single sacculus.

3. In *Scyllarus martensii* and *Scyllarides squammosus* a continuous, cord-like, capsulated spermatogenic mass traverses the vasa deferentia, whereas in *Parribacus tarcticus* the spermatogenic mass separates into distinct ampullae which become capsulated and are further provided with an enveloping sheath. This sheath between jacent ampullae becomes ribbon-like, twists, and ultimately forms short, doubly isted peduncles.

4. A secretion of the typhlosole in all three species surrounds the completed ermatophores and forms the homogeneous matrix.

5. Although these three species are anatomically similar, the spermatophores of *yllarus martensii* and *Scyllarides squammosus* resemble non-pedunculate spermaphores of the palinurids, the astacids, the homarids, and the nephropsids, whereas ose of *Parribacus antarcticus* more nearly resemble the pedunculate spermatophores certain pagurids.

6. Spermatophoric evidence above does not justify the inference that *Parribacus tarcticus* occupies a systematic position intermediate between the Macrura and the nomura.

INTRODUCTION

ALTHOUGH the origin and nature of certain rock lobster spermatophores have been investigated (Andrews, 1912; Matthews, 1954*a*), comparative udies have been neglected. The year-round availability in Hawaii of species hbracing three scyllarid genera (*Parribacus*, *Scyllarides*, *Scyllarus*) is proitious for such a study. Such a comparison should both extend our knowledge scyllarid biology and afford a clearer insight into the nature of their ermatophores.

Although Andrews's description (1912, p. 183) of the external spermatohoric mass of *Parribacus antarcticus* is correct, his reference to an 'internal . . . atrix enclosing tubular cavities' is erroneous (Matthews, 1954*a*). Calman's eneral statement (1909, p. 290) about the spermatophores of the Macrura is orrect, but his specific reference to the spermatophores of *Scyllarus* [sp. ?],

uarterly Journal of Microscopical Science, Vol. 95, part 2, pp. 205–215, June 1954.]

as will be shown subsequently, is incorrect. Since our knowledge of sc larid spermatophores is based largely on these conflicting accounts, need clarification is apparent.

METHODS AND TECHNIQUES

Both male and female *Parribacus antarcticus* (Lund), *Scyllarides squa mosus* (Milne Edwards), and *Scyllarus martensii* (Pfeffer) obtained fr fishermen and from the Honolulu Aquarium during the year 1953 were us in this study.

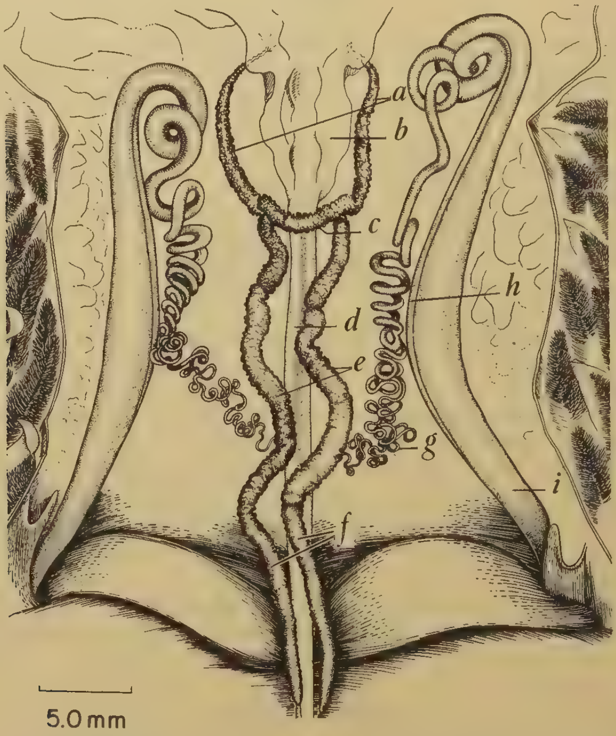


FIG. 1. Composite drawing of the male reproductive system as revealed by dissectio *Parribacus antarcticus*, *Scyllarides squammosus*, and *Scyllarus martensii*. *a*, anterior port of testes; *b*, pyloric region of stomach; *c*, transverse bridge; *d*, intestine; *e*, mid-portion testes; *f*, posterior portions of testes; *g*, proximal portion of vas deferens; *h*, hyaline l *i*, enlarged distal portion of vas deferens.

All dissections were made on living, non-anaesthetized specimens immer in sea water. The carapace was opened quickly and the internal organs ov lying the reproductive system were removed.

Right vasa deferentia of each species were either fixed in Bouin's fl washed in alcohol (70 per cent.), cleared in dioxane, embedded in Tissue (54–56° C.), sectioned at 10 μ, and stained in standard alum haematox and eosin; or they were fixed in Champy's fluid, washed in running w hours), dehydrated in alcohol, cleared in cedarwood oil, infiltrated with ene, embedded in Tissuemat (54–56° C.), sectioned at 4–6 μ, and stained iron haematoxylin. This latter method was also used for those ovaries and tes which were sectioned.

Left vasa deferentia of each species were immersed for 10–30 minutes in aqueous solution of toluidin blue (1:10,000), teased open in sea water, d their vitally stained contents studied and drawn.

OBSERVATIONS

ie morphology of the testes and the vasa deferentia

The mature testes of these three species are macroscopically similar. Freed overlying tissues all resemble an elongate H, the right and left sides being ned by a transverse bridge (fig. 1, *c*) immediately posterior to the junction the pyloric region of the stomach (*b*) and the intestine (*d*). Occasionally the terior portions of the testes (*a*) bifurcate, although the posterior portion (*f*), nich lies contiguous with the digestive tract (*d*), is unaltered. The testis is mposed of a highly coiled, continuous, thin-walled tube into which in- umerable sacculi (fig. 6, *a*) open.

The mature vasa deferentia emerge from the mid-portions (*e*) of the testis. hey are macroscopically dissimilar in the three species. Moreover, the right d left proximal portion (*g*) of the vas deferens of a single specimen may vary much as those of different species. In all species the enlarged distal portion which opens on the coxopodite of the fifth pereiopod is less varied. This ortion in all three species is marked by a distinct hyaline line (*h*).

Even when the vasa deferentia are quickly exposed in dissection, they fail exhibit muscular contraction. This fact, together with their opacity, pre- nts one from observing the mechanics of the development of the spermato- nores. However, by sectioning and vital staining, the otherwise obscure fects of contractions of the unaltered vasa deferentia are discernible.

istology of the testes and the vasa deferentia

Sections through immature sacculi of *Parribacus antarcticus* (fig. 2, A) osely resemble those of *Scyllarus martensii* (fig. 2, B). In both species, cculi in all stages of development are observed. Although there are small fferences in saccular size, these vary as much within a species as between ose of different species. From observations of hundreds of sacculi in both *arribacus antarcticus* and *Scyllarus martensii* the author is of the opinion that ose of *Parribacus antarcticus* are slightly larger. The connective tissue (*a*) hich envelops the sacculi in both species is approximately of equal thickness μ). The similarity of sacculi is further attested by the general likeness of e germinal epithelium (*b*) and the primary spermatocytes (*c*). In both species ese cells are of approximately the same size (20 μ). In addition the immature cculi of both species exhibit sustentacular-like cells (*d*) with indistinct toplasm and strongly chromophil nuclei, but their origin and method of

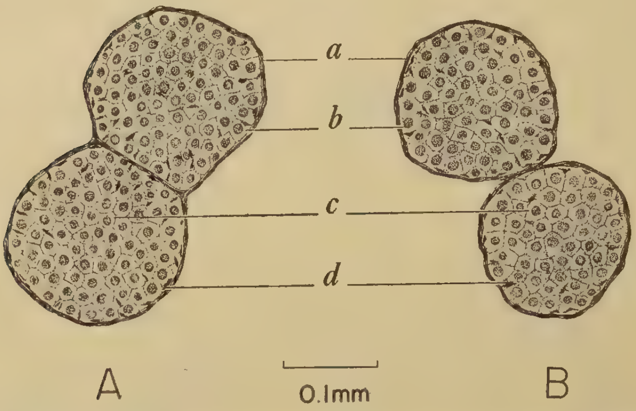


FIG. 2. Camera lucida drawing of sections through immature sacculi of A, *Parribacus antar*
cus, and B, *Scyllarus martensii*, showing: *a*, connective tissue; *b*, germinal epithelium;
primary spermatocytes; *d*, sustentacular-like cells.

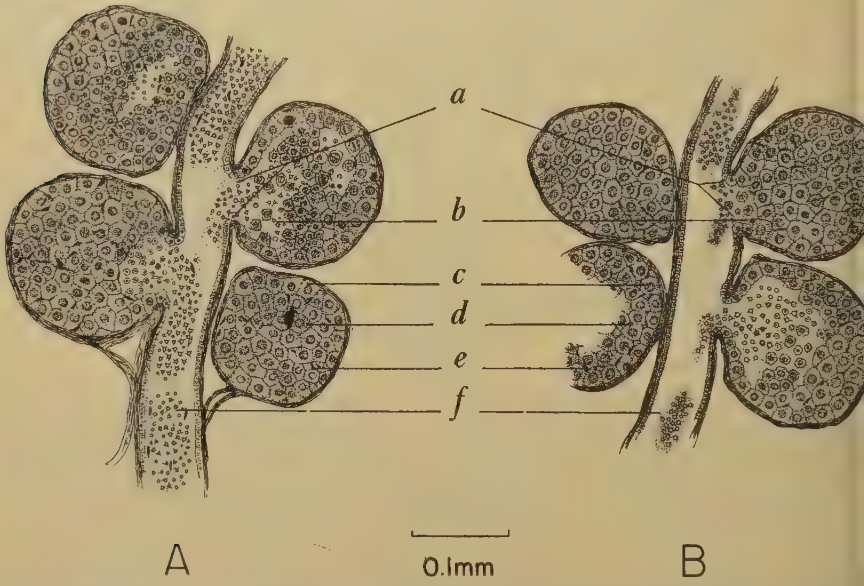
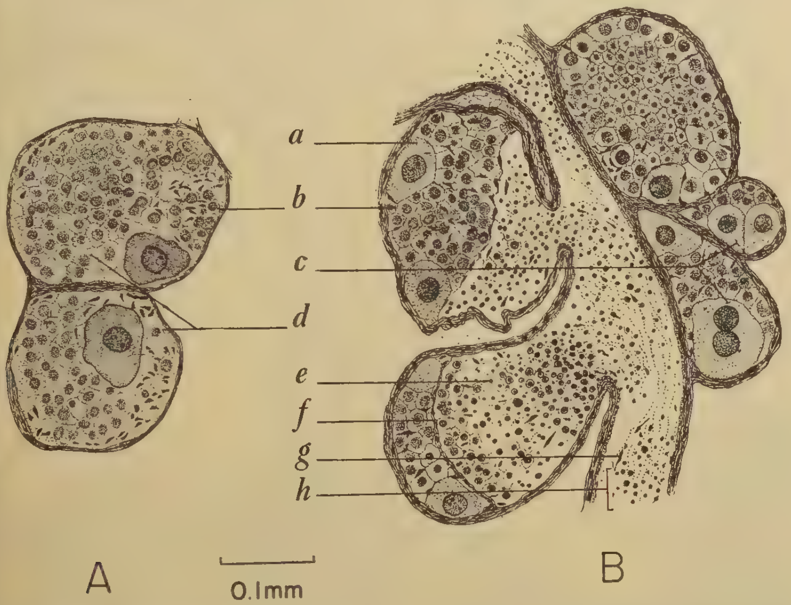


FIG. 3. Camera lucida drawing of sections through mature sacculi of A, *Parribacus antarcti*
and B, *Scyllarus martensii*, showing: *a*, openings into collecting tubules; *b*, spermati
c, germinal epithelial cells; *d*, primary spermatocytes; *e*, sustentacular-like cells; *f*, clump
of metamorphosing spermatids.

ultiplication is obscure. When first observed, these cells lie contiguous with e germinal epithelium, an association which suggests but does not prove eir origin. Concurrent with their increase in number, the meiotic divisions the spermatocytes produce large numbers of spermatids.

Although many mature sacculi are seen, few reveal their actual opening to the collecting tubule (fig. 3, *a*); yet upon these sections in both *Parribacus tarcticus* (fig. 3, A) and *Scyllarus martensii* (fig. 3, B) depends our underanding of saccular activity. Concurrently with the metamorphosing of the permatids (*b*), the germinal epithelial cells (*c*) renew their mitotic activity and gain produce primary spermatocytes (*d*), which, as they fill the sacculi, expel he metamorphosing spermatids. This rhythmical activity of the germinal pithelium probably accounts also for the clumping of the metamorphosing permatids in the tubule, each clump (*f*) representing the extruded contents f a single sacculus. It was not determined whether the sustentacular-like ells (*e*), some of which accompany the metamorphosing spermatids into the ubule, aid in the clumping process or simply serve as nutritive cells.



IG. 4. Camera lucida drawing of sections of A, immature sacculi, and B, mature sacculi of *cyllarides squammosus*, showing: *a*, oocyte-like cell; *b*, germinal epithelial cells; *c*, oocyte-like ells dividing; *d*, primary spermatocytes; *e*, spermatids; *f*, delimiting wall of sustentacular-ke cells; *g*, sustentacular-like cells in tubule; *h*, clumping of metamorphosing spermatids in tubule.

Although the sacculi of *Scyllarides squammosus* (fig. 4) are in general like hose of *Parribacus antarcticus* and *Scyllarus martensii*, dissimilarity is at nce apparent by the anomalous appearance of bizarre, oocyte-like cells (*a*). Their presence suggests an ovitestis possibly resulting from certain castraion-parasites (*Altheles*, *Chlorogaster*, *Peltogaster*, &c.); however, examination reveals neither evidence of castration-parasites nor secondary sexual characte These inquiline, oocyte-like cells are further misleading because in osmiu tetroxide preparations they exhibit lipoid cytoplasmic inclusions comparabl to those of normal oocytes. This evidence simply attests their general oocyt like appearance, but further study discloses their true spermatogene function.

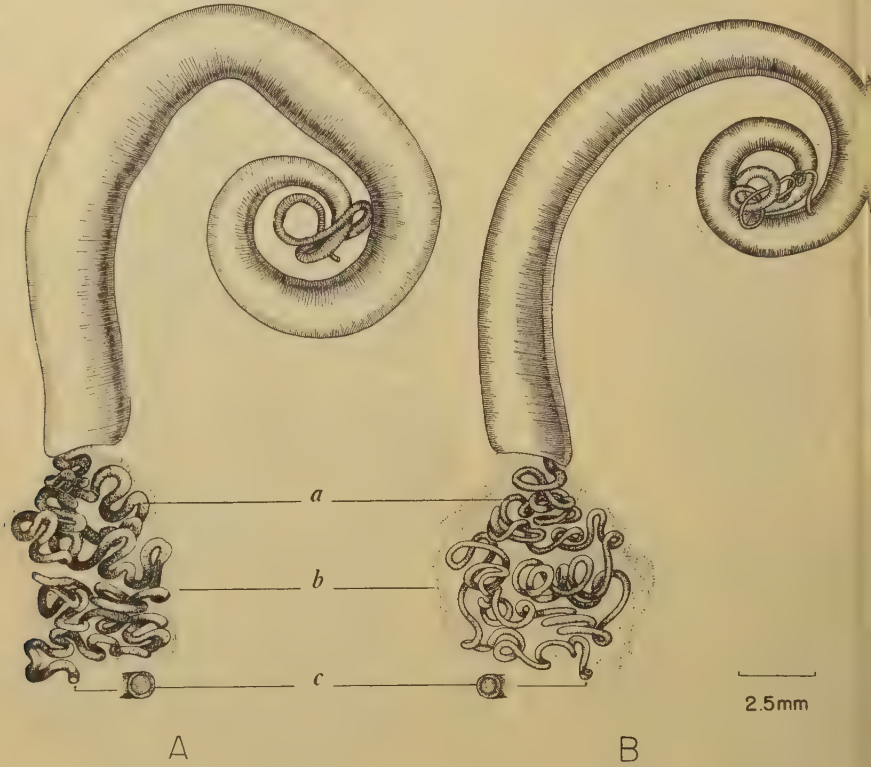
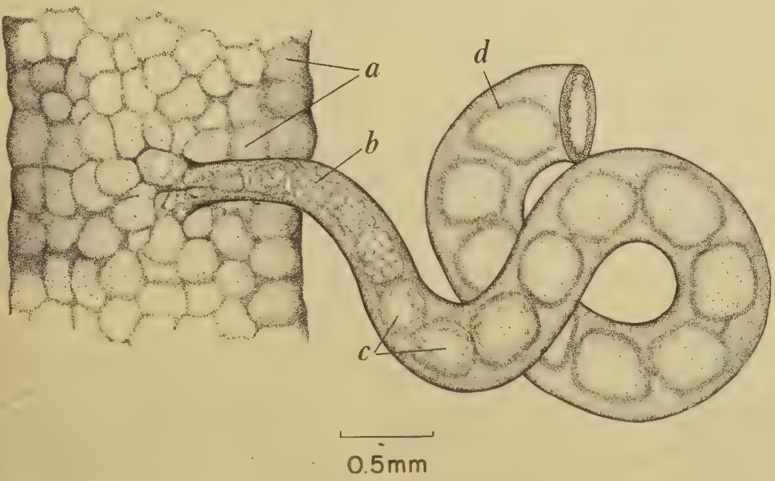


FIG. 5. Extruded masses from vitally stained portions of the enlarged vasa deferentia of *Scyllarides squammosus*, and B, *Scyllarus martensii* showing: *a*, continuous, thread-l spermatophore; *b*, homogeneous matrix; *c*, capsule (enlarged).

Apparently in this species each germinal epithelial cell (*b*) possesses t potentiality for the production of primary spermatocytes, but actually t function is restricted to comparatively few cells. While other germi epithelial cells remain unaltered, these few increase greatly in size (up 135 μ), divide (*c*) and redivide mitotically, subsequently filling the saccu with primary spermatocytes (*d*). These, as in the two species previou described, undergo meiotic divisions, filling the sacculi with spermatids (

In this species also, then, the rhythmical expulsion of the metamorphosi spermatids is evidenced as new primary spermatocytes are formed. Wher in *Parribacus antarcticus* and *Scyllarus martensii* the new crop of prim



IG. 6. Vitally stained portion of the testis and proximal vas deferens of *Parribacus antarcticus*, howing: *a*, sacculi of testis; *b*, undifferentiated spermatogenic mass; *c*, moniliform portion of spermatogenic mass; *d*, distinct ampulla of sperm with thick-walled capsule.

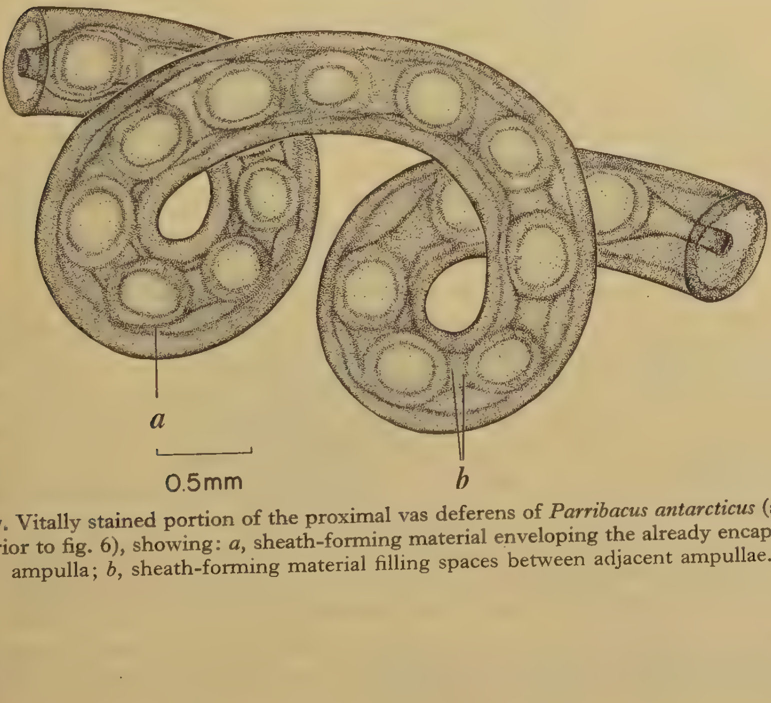


FIG. 7. Vitally stained portion of the proximal vas deferens of *Parribacus antarcticus* (slightly posterior to fig. 6), showing: *a*, sheath-forming material enveloping the already encapsulated ampulla; *b*, sheath-forming material filling spaces between adjacent ampullae.

spermatocytes is not sharply marked off from the metamorphosing spermatid in *Scyllarides squammosus* they are delimited by a thin wall of sustentacula like cells (*f*). As in the two species previously described, the fate of th sustentacular cells (*g*) which accompany the metamorphosing spermatids in the collecting tubule is obscure.

Vital staining of the vasa deferentia

Vital staining of the vasa deferentia reveals in *Scyllarides squammosus* (fi 5, A) and *Scyllarus martensii* (fig. 5, B) a continuous, thread-like spermatopho (*a*). In these two species the spermatophoric mass, probably by muscul contractions of the vasa deferentia, is both moved along and moulded in co formity to their lumina, which are circular in transverse section. Moreove

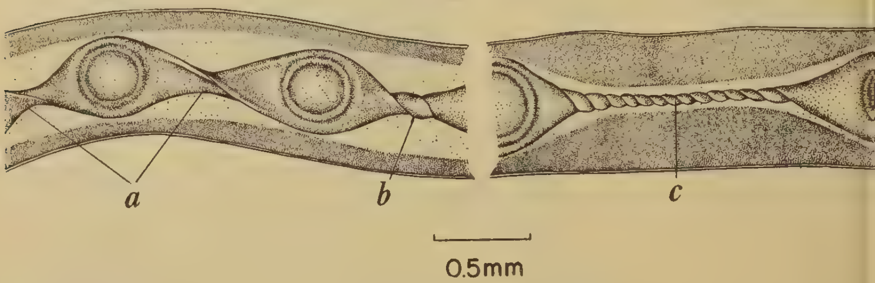
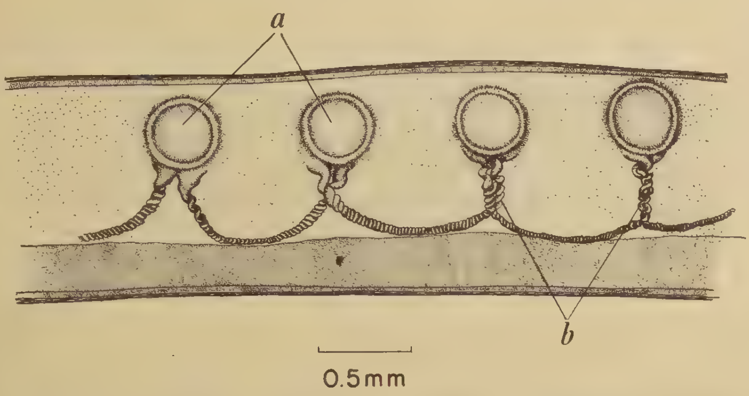


FIG. 8. Vitally stained portions of the proximal vas deferens of *Parribacus antarcticus* (slight posterior to fig. 7), showing: *a*, ribbon-like sheath between adjacent ampullae; *b*, *c*, chalaz like twisting of sheath.

as the walls of the vasa deferentia thicken, a secretion from crypt-like fol of the epithelium surrounds the cord-like mass and forms the capsule (*c*). these two species, as the lumina of the vasa deferentia enlarge, the encapsulate cord-like masses become highly coiled and folded; however, they remai continuous throughout. As they traverse the enlarged portion of the va deferentia these masses are enveloped by a secretion of the typhlosole. Th secretion forms the homogeneous matrix (*b*).

Since vital staining of the vas deferens of *Parribacus antarcticus* has bee discussed in a previous paper (Matthews, 1954*a*), only those points pertine to this comparative study will now be summarized. By vital staining (fig. the undifferentiated spermatogenic mass (*b*) is seen emerging from the saccu of the testis (*a*). This mass, probably by contractions of the walls of the prox mal portion of the vas deferens, is moved along, made moniliform (*c*), ar finally broken into distinct ampullae. A secretion from the crypt-like folds the epithelium surrounds each ampulla and forms a thick-walled capsule (A new secretion from the epithelial cells lining the vas deferens (fig. envelops the already encapsulated ampullae (*a*) and fills the spaces betwe them (*b*). This secretion forms the sheath. This sheath (fig. 8) betwe adjacent ampullae becomes ribbon-like (*a*), probably as a result of muscul contraction of the walls of the vas deferens; and as the ampullae traverse t



IG. 9. Vitally stained portion of the distal vas deferens of *Parribacus antarcticus*, showing: *a*, raised ampullae; *b*, short, doubly twisted peduncles.

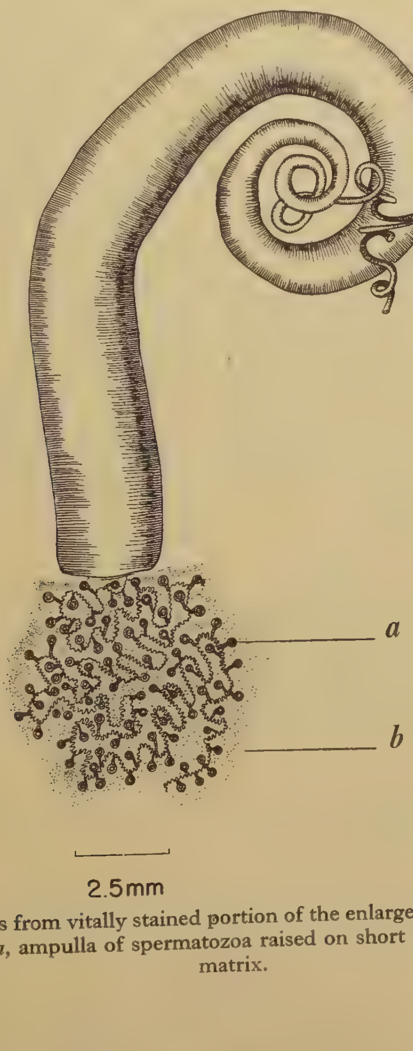


FIG. 10. Extruded mass from vitally stained portion of the enlarged vas deferens of *Parribacus antarcticus*, showing: *a*, ampulla of spermatozoa raised on short peduncles; *b*, homogeneous matrix.

coiled vas deferens, this ribbon-like sheath becomes twisted (*b*, *c*) so as resemble chalazae. Continued twisting (fig. 9) causes these to entwin raising the ampullae (*a*) on short doubly twisted peduncles (*b*). As in the tw previously described species, a secretion from the typhlosole of the enlarge distal portion of the vas deferens (fig. 10) now envelops the completed spermatophore (*a*) and forms the homogeneous matrix (*b*). Whereas *Scyllarid squammosus* is set apart by its saccular activities, *Parribacus antarcticus* is s apart by the activity of its vasa deferentia.

Discussion

All recorded observations indicate that the palinurids (with the possib exception of *Jasus lalandii*, von Bonde, 1936), the astacids, the homarids, an the nephropsids produce continuous, highly convoluted spermatophore embedded in homogeneous matrices (*Panulirus interruptus* (Allen, 191 Fasten, 1917; Wilson, 1948); *P. argus* (Crawford and De Smidt, 1923); *P penicillatus* (Matthews, 1951); *Potamobius trowbridgii* (Andrews, 1931 *Homarus americanus* (Herrick, 1895); and *Enoplometopus occidentalis* (Matthew 1954*b*)). These recorded observations are in accord with Calman's (190 general statement that 'in most Macrura the spermatozoa are enclosed in sheath, forming a continuous cord-like mass'. Moreover, he states that 'i the Anomura this is broken up into separate spermatophores attached by on end in a row on a strip of membrane'. The Macrura therefore produce non pedunculate spermatophores whereas the Anomura produce pedunculat spermatophores (see Mouchet, 1930, 1931; Matthews, 1953).

The spermatophores of *Scyllarides squammosus* and *Scyllarus martens* clearly belong to the non-pedunculate Macrura, whereas those of *Parribac antarcticus* appear intermediate between the non-pedunculate Macrura an the penduculate Anomura. However, this is insufficient evidence th *Parribacus antarcticus* is intermediate between these two groups. Anatomica embryological, and serological evidence should accompany such evidenc from the spermatophores, and this should be obtained from many specie representing many genera.

The results of this study justify two conclusions: (1) species of otherwis closely related genera do not necessarily produce similar spermatophores, an (2) the origin of peduncles may have been only the fortuitous occurrence mechanical and physiological activities of the vas deferens, inasmuch as thos of *Parribacus antarcticus* fail to elevate the ampullae for the subsequent dispersal of the spermatozoa.

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An Investigation of the Innervation of the Thymus Gland of the Common Mouse, *Mus musculus*

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With two plates (figs. 2 and 3)

SUMMARY

The anatomical relations of the thymus gland in mice have been studied in serial sections throughout the entire thoracic and posterior cervical regions. No evidence was obtained that the thymus has a secretory function under the control of the nervous system, and no nerve fibres or endings were observed in the proximity of the thymocytes, Hassall's corpuscles, or reticular cells. The thymic innervation resembles that of the lymph nodes rather than that of the adrenal gland or of the spleen and the neuro-histological picture was unchanged during accidental or age involutions. The thymic innervation consists entirely of autonomic vasomotor nerves originating in an outlying sympathetic plexus—the 'thymic plexus', and it is suggested that thymus functions are controlled by vasodilatator and vasoconstrictor mechanisms with the possible aid of thymotropic hormones carried in by the blood-stream. The thymus may aid in the regulation of the lymphocyte content of the blood, acting as a lymphocytic reservoir instead of as a lymphocytopoietic organ.

The source of thymic innervation could not be determined solely by anatomical methods and the majority of current histological techniques were found to be unsuitable for differentiating between nervous and non-nervous argyrophilic fibres. The basic techniques of Holmes and Romanes appeared to satisfy the necessary criteria and Steedman's ester wax method was successfully used in conjunction with these techniques.

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INTRODUCTION

THE incomplete state of our knowledge of thymic function and structure is obvious from only a brief glance at the literature. Haemopoietic, incretory, and reticulo-endothelial functions have all been suggested as possible activities of the thymus gland but none has been demonstrated conclusively. Sufficient stress has not been laid on a determination of the controlling mechanism of this gland without which any assessment of its functions remains

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incomplete. Two possible types of control may be postulated: (1) nervo
control, either by means of components from both spinal and autonom
systems or by autonomic components only, and (2) hormonal control, throug
the blood-vessels with or without the aid of nervous elements. It was felt that
knowledge of the characteristics and disposition of the nervous elements in t
thymus would give a valuable pointer to the type of function to be expecte
and therefore a study of the innervation was chosen as the subject of th
present investigation.

The literature on thymic innervation is very scanty and somewhat confuse
The older anatomists such as Luschka, Kölliker, Krause, and Poirier and Charp
as quoted by Sjölander and Strandberg (1915) all suggest that the thymic nerv
in man are vascular nerves, but all are vague as to their origin; however, Friedl
ben (as quoted by Sjölander and Strandberg, 1915) describes them as comin
from the sympathicus. Jossifow (1899), Bovero (1899), Sjölander and Strandbe
(1915), Braeucker (1923), and Hammar (1925) described fibres arriving at t
capsule of the thymus of human foetuses. Hammar describes them as comin
from the vagus, Jossifow from the vagus and plexus mammarius inter, an
Bovero, Sjölander and Strandberg, and Braeucker from both vagus and sym
pathicus, and indirectly from the phrenic and ansa hypoglossi. Cordier an
Coulouma (1934) describe similar fibres in the dog, cat, and rabbit comin
from the vagus by its thoracic segment, recurrent, middle, and inferior cardi
nerves, and from the sympathetic through its inferior cervical and superi
thoracic ganglia and the middle and inferior cardiac nerves. All these exam
nations were purely anatomical and did not determine which of the nerv
fibres arriving at the capsule actually penetrated into the cortical or medulla
tissue. The only neuro-histological work was that of Pines and Majman (1929
but, as will be shown in this paper, the techniques which they used were n
suitable for differentiating between argyrophil fibres and fine nerve fibres. It
of interest to note that most of the structures they considered as nervo
elements have been correctly described by Smith and Ireland (1941)
belonging to the argyrophil system. Selye (1948) summarizes the prese
inadequate knowledge of thymic innervation in man by stating that 'there a
only very fine nerve filaments in the thymus. These are derived from t
sympathetic and vagus. Branches from the descendant hypoglossus and phren
nerves reach the capsule but fail to penetrate into the thymus.' These statemen
are unaccompanied by either concrete evidence or references to original wor
Apparently this is assumed to be common knowledge even although it
unproved.

The research presented in this paper was therefore undertaken with t
following aims:

1. To determine which of the nerve fibres in the vicinity of the gland act
   ally penetrate into the gland tissue.
2. To determine the course of nerve fibres within the gland and to disti
   guish these from the reticular fibres present.

3. To determine the types of nerve components present and the relation of their endings to the thymocytes, the Hassall's corpuscles, and the so-called reticular cells, and therefore to establish which cells might be considered to have a secretory function.
4. To determine whether or not the character of the innervation changes during involutions owing to ageing, accidents, or cyclical changes.
5. To compare and contrast the innervation of the thymus with that of endocrine organs such as the adrenal and of haemopoietic organs such as the spleen and lymph nodes.

MATERIAL

The common mouse, *Mus musculus* Linn., was chosen for the present study ecause of the shortness of the breeding cycle, which makes it possible to bserve many generations in a limited time. An agouti strain with a low tumour cidence was kept as it was felt that agouti mice tended to be more normal an the much modified albino variety. The mice were bred under uniform nditions of housing and feeding; draught-free cages were used and the food nsisted of commercial rat cake (with 14 per cent. dried milk) as supplied to e Rowett Research Institute. Accurate records were made of the ages and rentage of all animals.

Killing by a blow on the head was found to be impracticable as this usually used either extreme congestion of the anterior thoracic blood-vessels or emorrhage in the region of the thymus. All mice were therefore anaesthezed with ether and dissected before death.

METHODS

As the techniques for demonstrating nervous elements played an important art in this investigation they will be discussed in some detail.

natomical methods

Dissection of the neck and thorax was done under warm physiological line with the aid of fine iridectomy instruments, a surgical cautery set, and a inocular dissecting microscope with total magnification × 61. Knisely's (1936) ansillumination method was used in examination of the extirpated thymus land.

In a few instances the maceration method of Sjölander and Strandberg 915) was used also as a preliminary to examination under the binocular icroscope. This consisted of removing the entire cervico-thoracic region, xing it in formalin, and then allowing it to macerate in a mixture of one part f glacial acetic acid, four parts of glycerine, and ten parts of distilled water. he time (several months) given by Sjölander and Strandberg, who applied e method to human foetuses, had to be modified considerably when dealing ith mice. It was found that 2 hours in the macerating fluid was sufficient to issolve away the surrounding muscles and connective tissues and to leave the nerve fibres in the vicinity of the gland intact. This method, however, prov somewhat difficult to control, owing to the extremely soft nature of the thym tissue, which tended to disintegrate rapidly.

Histological methods

The chief problem of histological examination was to discover a techniq suitable for demonstrating the finest nerve fibres and endings, and for di tinguishing these clearly from reticular elements. The techniques used ca conveniently be considered in three main categories, viz. (1) freezing tec niques, (2) celloidin techniques, and (3) paraffin wax and ester wax technique It was found that not one of the methods used could with absolute certain distinguish the argyrophil fibres of the supporting matrix from true ner fibres. The best techniques were those of Holmes (1943) and Romanes (195 but these still require to be perfected for postganglionic autonomic fibres. each case the thymus gland was removed with the aid of a cautery set befo the death of the mouse.

Freezing techniques. Thymus glands, either directly or after embedding gelatin, were frozen to the holder of the freezing microtome. Both metho gave a poor histological picture, owing to insufficient firmness in the 'holdin of the loosely packed thymocytes (see fig. 2, E). Several well-known staini methods were tried with the results shown below.

Weddell's silver method (1941) proved useless for the thymus owing to t large amount of reticulum present. It stained all but the finest nerve fibres b also stained the fine reticular fibres of the thymic matrix. Fig. 2, E, shows su reticular fibres proceeding inwards from the capsule.

The Gros-Schultze method failed to distinguish with certainty between t nervous and non-nervous argyrophil fibres, for although the toning in go chloride had the expected effect of turning the larger collagen fibres purp instead of black it had no differentiating effect on the small argyrophil fibr

Del Rio-Hortega's double impregnation method did not improve the staini as the same difficulty arose. Although Del Rio-Hortega suggested that stronger solution of $AgNO_3$ would prevent connective tissue from staining, these experiments variations in the strength of the $AgNO_3$ were found produce no appreciable difference in the staining. This was probably due the lack of a corresponding increase in the ionization of the silver, and hen of the rapidity with which the nerve fibres could become impregnated.

Celloidin techniques. The above methods of Weddell, Gros-Schultze, a Del Rio-Hortega were used after preliminary embedding in celloidin a sectioning on a sledge microtome. The celloidin embedding improved t general cytological picture and prevented most of the shrinkage usua obtained in freshly fixed, non-embedded material.

Paraffin wax and ester wax methods. These methods made it easier to secti in series thoracic and cervical contents which had been removed *in toto*. Su sections served as an additional check on the value of the various staini techniques used, as in each section samples of several kinds of tissues w nd including cardiac muscle, connective tissue, non-medullated nerves, l oesophageal epithelium. The thymus was consistently more difficult to l with than the adjacent tissues.

Paraffin wax of melting-point 56° C. was used and sections were cut at from to 15 μ. Even with very carefully controlled embedding the resulting sections ded to show shrinkage, but the least shrinkage was found in glands cleared chloroform.

Steedman's ester wax method (1947) gave results equivalent to celloidin thods with the added advantage of rapid serial sectioning. The shrinkage e to paraffin embedding was largely eliminated and there was no adverse ect on the silver staining.

In all, approximately 100 thymus glands from mice of varying ages were bedded in either paraffin or ester wax, serially sectioned, and examined. ntrol glands from each of the age groups were fixed in Helly-Zenker, bedded, sectioned, and stained either with Heidenhain's haematoxylin and sin, or with Azan. Glands from litter-mates were then prepared in various ys for the study of the nervous elements. Two main types of silver technique re employed, block methods and slide methods. In block methods the ymus gland and surrounding structures were stained in bulk previously to bedding in paraffin or ester wax, and in the slide methods the tissues for amination were embedded, sectioned, and fixed on slides before being ined.

The block methods used were those of Ramon y Cajal (Willard, 1935–6), elschowsky, and Pines and Majman (1929). As in all block methods the ining was difficult to control and results tended to be uneven owing to the equal penetration of the reagents used. In particular the silver nitrate nded to become deposited around the periphery of the tissues if they were owed to remain in it for a length of time sufficient to permit penetration to e centre. Fig. 1 shows this clearly. The time taken for the silver to be de- sited on the argyrophil fibres is a good indication of the rate of penetration the reagents used in block methods in general. All block methods, even when ed on glands of only 5 mm. in thickness, gave haphazard results. Many of the sults recorded by investigators may owe their lack of constancy to this type technique, which is insufficiently critical for an analysis of innervation.

In general, it was discovered that none of the above methods could be relied to distinguish absolutely between the finest nerve fibres and the so-called gyrophil fibres'. In particular the methods of Pines and Majman (1929) ere found to be unreliable. It can be seen from a careful survey of sections epared by these methods (fig. 2, A and B) that fine connective tissue fibres ve stained well, but no nervous elements are visible. This startling fact serves serious consideration, as the entire study of the innervation of the ymus by Pines and Majman is based on this type of technique.

Bielschowsky's acetic acid treatment was also found to be insufficient to colorize the argyrophil fibres, as was pointed out by Nonidez (1936) working a variety of organs other than the thymus. However, I cannot agree entirely

with Nonidez when he states that 'with the original Cajal method (for bloc
the connective tissue fibres do not appear impregnated after a variety
fixatives (chloral hydrate, pyridine, formalin, alcohol, or alcohol with ot

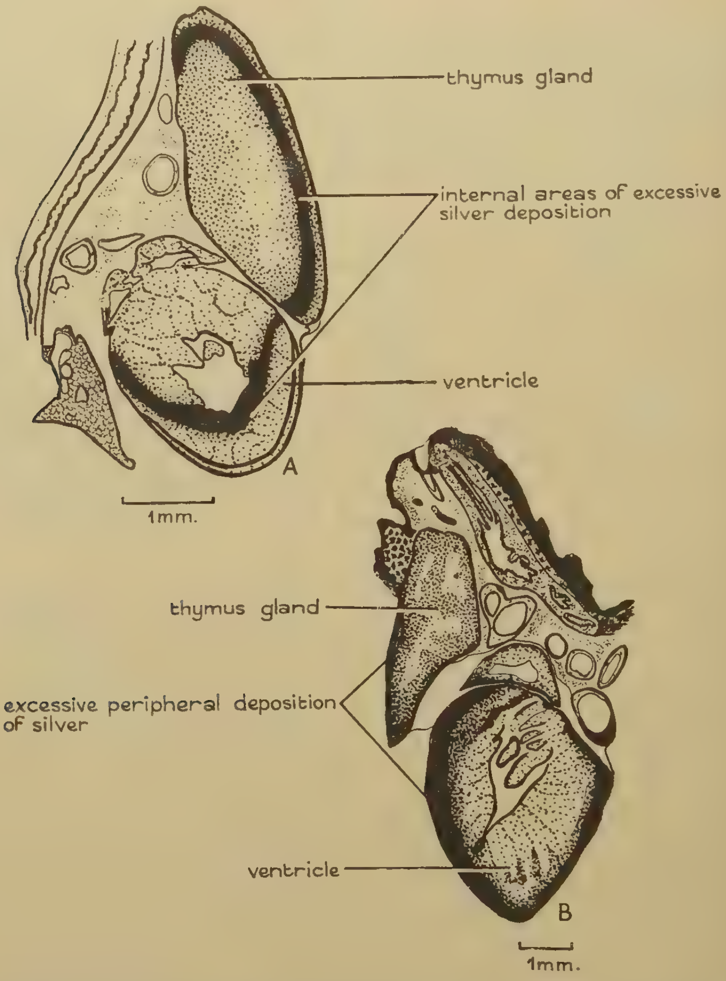
thymus gland
internal areas of excessive silver deposition
ventricle
A
1mm.
thymus gland
excessive peripheral deposition of silver
ventricle
B
1mm.

Fig. 1. Longitudinal sections through portion of cervico-thoracic contents of mouse. A,
day male mouse. Pines and Majman block method. Ester wax. B, 25-day female mou
Ramon y Cajal (Willard). Ester wax.

substances such as veronal, urethane, chloral hydrate, somniphene, ammon
&c.) but in material fixed with the alcohol ammonia mixture they may occasio
ally stain if too much ammonia has been added'. I found that with chlo
hydrate, pyridine, formalin, or alcohol the finest argyrophil fibres of the thym
did become impregnated with the silver nitrate (fig. 2, c and d).

Slide methods avoided the difficulty of uneven penetration of chemica

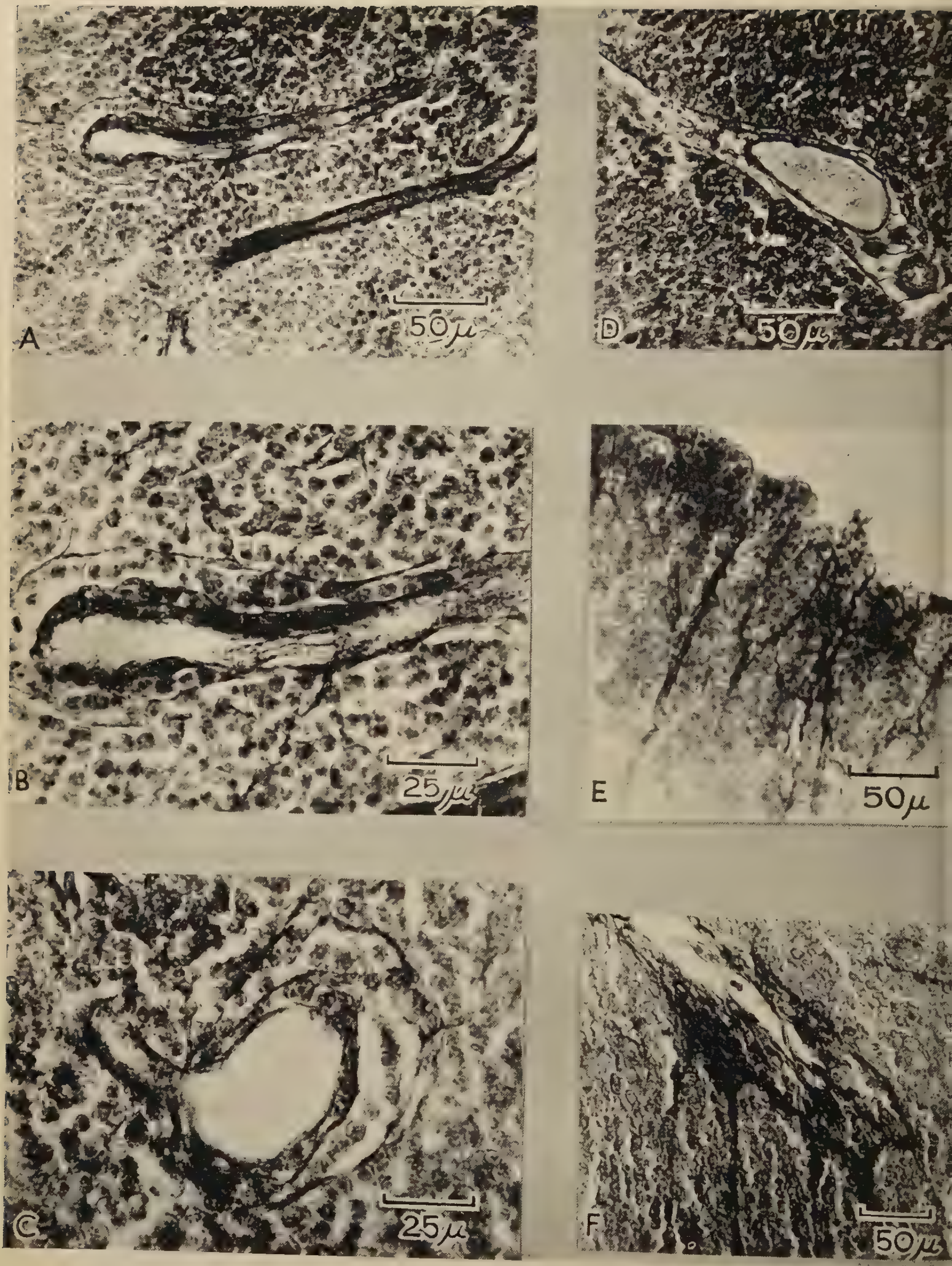

Fig. 2

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e technique which promised best was that of Bodian (1936), but as the only
mple of 'protargol' which was available was inactive, the impregnations were
t good and the method had to be abandoned. The methods of Young (1939),
blin (1944), and Evans (1947), who also utilized 'protargol', were not suc-
ssful for the same reason. Young (1939) states, however, that this method in
original form only occasionally stains the very finest nerve fibres. Holmes's
cellent treatise (1943), which has done much toward placing silver staining
on a sound scientific basis, suggests that silver oxide and silver carbonate
ve 'preparations closely resembling those of the Protargol method. .. .' Silver
rbonate was therefore tried but was found to be insufficiently constant, as
deed has already been pointed out by Holmes himself. His ammoniacal
ver method could not be relied on to demonstrate finer nerve endings.

In a personal communication, since published (1950), Romanes suggested a
de method based on Holmes's findings which he had found satisfactory for
monstrating fine nerve fibres and endings in paraffin sections of a variety
tissues, and for differentiating them from argyrophil fibres and collagenous
ructures. A slightly modified form of the Romanes method was therefore
ed, the pH of the freshly prepared silver chloride solution being adjusted
th a Beckman pH meter to exactly 9·0. The pH reading was again taken at
e end of staining and the sections lifted out when a pH of 8·0 was obtained,
at this time the sections had attained the light brown colour indicative of
ver reduction by the tissue itself (Holmes, 1943; Romanes, 1950). The
riod in the 2 per cent. oxalic acid solution was reduced to 1 minute as a
nger period increased the intensity of staining of the connective tissue fibres.

ital staining methods

Intravitam injections of Gurr's vital methylene blue were used in the pro-
rtion of 1 ml. of a 0·1 per cent. solution in physiological saline per gramme of
dy-weight of mouse. This dose was injected either subcutaneously, intraperi-
neally, intrathoracically, or intracervically. Injections given intrathoraci-
lly were found to be unsatisfactory as the surface of the thymus became
o deeply stained to allow any vessels and nerves to be seen clearly, even

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FIG. 2 (plate). Photomicrographs of sections of the thymus gland of the mouse to show
gyrophil fibres which simulate nerve fibres.
A, thymic medulla of 11-day ♀ mouse showing fine reticular fibres surrounding blood-
ssels. Pines and Majman silver method. Ester wax.
B, portion of upper vessel in A enlarged to show reticular structure (centre of field) simu-
ting nerve terminal arborization owing to tangential cut.
C, thymic medulla of 11-day ♀ mouse showing direct connexion of reticular fibres round
ymic artery and vein with the capsular fibre in the septum at upper left of picture. Ramon y
ajal (Willard). Ester wax.
D, same section as C, showing 'double ring' of argyrophil (non-nervous) fibres surrounding
all blood-vessel.
E, frozen section through thymic medulla of 90-day ♂ mouse showing reticular fibres
oceeding inwards from capsule. Weddell.
F, thymic medulla of 28-day ♀ mouse showing fibres surrounding blood-vessels and
nnecting with fibres of supporting network. Azan. Photographed with yellow filter.

with transillumination. This method was therefore abandoned. Intraperi neal injections, repeated after an interval of a day, were found to prod active staining of the mediastinal lymph nodes although thymic staining w still poor.

Intravital perfusion through the ventricles of 0·1 per cent. vital methyle blue after warm physiological saline tended to overload the liver cells with c particles before the thymus cells had time to pick up the stain.

Supravital staining by immersion of the thymus in a 0·1 per cent. s pension of the vital dye in physiological saline immediately after remo under deep ether anaesthesia was found to be the most satisfactory method the three, as the thymic cells took up the stain slightly.

After intravitam perfusion or injection the thymus was exposed a examined during life under a high-powered binocular dissecting microsco by transillumination. The thymus was then removed, transferred to a wa cavity slide, and examined rapidly under the compound microscope w the aid of transillumination. In general results were inconclusive owing to t faint staining and the general hindrance to visibility by the large numbers thymocytes.

After supravitam staining the gland was fixed in ammonium molybdate preserve the stain, rapidly dehydrated and cleared in 'cellosolve' (ethyle glycol monoethyl ether), embedded in ester wax, and sectioned. The peri in 'cellosolve' had to be curtailed as this chemical was found to have a c colorizing effect.

Nerve section methods

The impossibility of transecting the finest branches of either the vagus cervical sympathetic under surgical conditions, together with the lack of su able histological techniques to demonstrate nerve degeneration areas, effe tively ruled out nerve section methods as a means of studying the innervati of the thymus.

RESULTS

Central connexions

The first regions to be examined in the search for central nervous connec ions were those of entering and outgoing blood-vessels. It was felt that ner bundles would be most likely to take the vascular pathways and would ther fore be found in the same septa as the arteries and veins. There are two su

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FIG. 3 (plate). A, ventral cervico-thoracic dissection of 36-day-old male mouse showing th mus typical of adolescence. Half of ventral thoracic wall is deflected to right and half to le

B, photomicrograph of nerve bundle in cardiac muscle. Same section as fig. 2, C and

C, photomicrograph of vagus nerve from same series as fig. 2, C and D.

D, photomicrograph showing thymic plexus with surrounding adipose tissue. Anter portion of antero-ventral interlobular septum is seen in lower part of photograph. From sam series as fig. 2, C and D.

E, photomicrograph of antero-ventral interlobular region of 25-day ♀ mouse thym showing incoming nerve fibre at lower right and portion of thymic plexus at upper rig From same series as D.

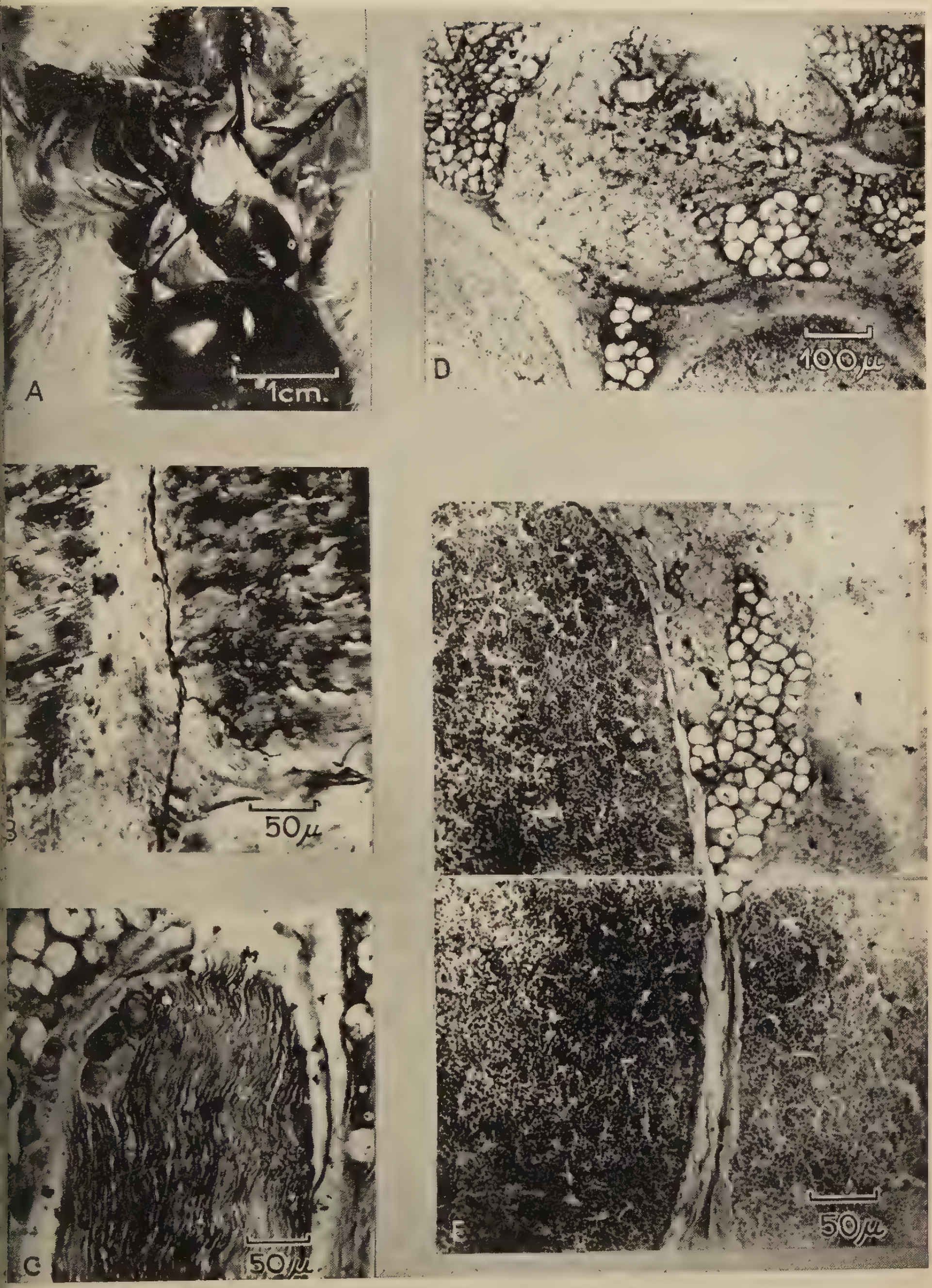

Fig. 3

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gions in each lobe of the thymus gland. The first, illustrated in fig. 3, A, is ntro-lateral in position and receives the main thymic artery from the internal ammary artery. The main thymic vein leaves from this area and joins the ternal mammary vein. The second region is much less constant in position ut is usually situated antero-dorsally. At this point a small artery and vein

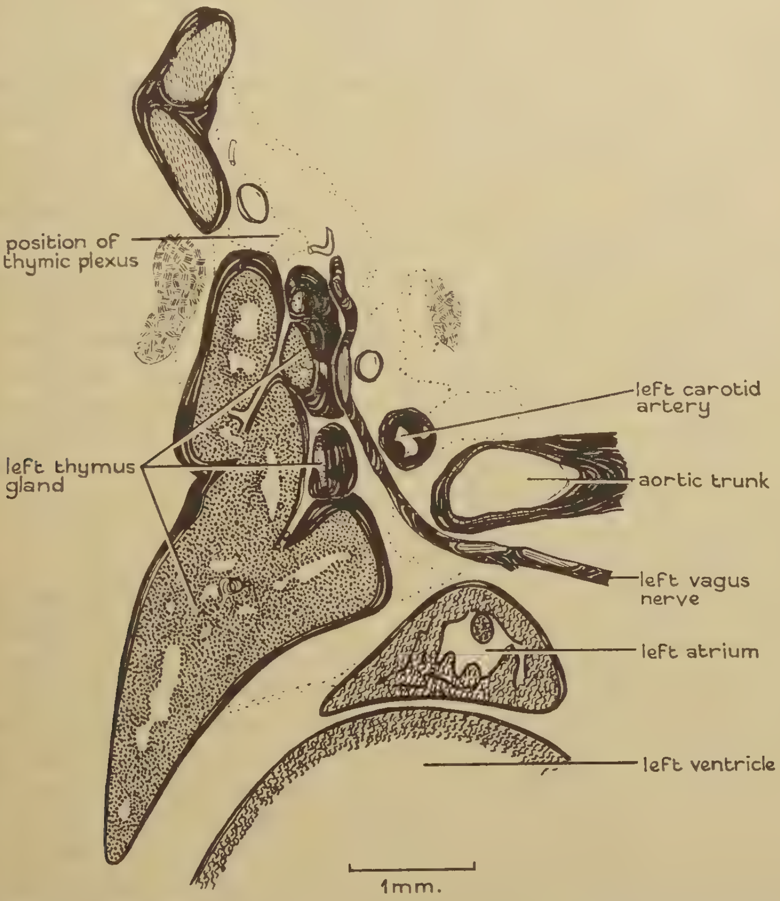

FIG. 4. Longitudinal slice, 115 μ thick, through anterior thoracic contents. From reconstruction by Smith's 'cellophane' method of twelve sections from same series as fig. 1, B.

are seen entering and leaving respectively. The artery is a branch of the inferior thyroid artery and the vein leads into the inferior thyroid vein. The relative sizes of these two pairs of vessels were found to vary somewhat but the condition outlined above was the one most frequently found. Serial sections of the entire cervical and thoracic contents taken through transverse, longitudinal, and horizontal planes give a true picture of the exact relative positions of the structures concerned. Fig. 4 is a reconstruction made by Smith's cellophane method (1938) from a series of longitudinal sections, and shows clearly the course of the vagus and the position of the sympathetic plexus in relation the thymus gland. However, the determination of the central connexions the thymic nerves proved to be one of some difficulty as the only fibres like to be concerned were very fine and non-medullated. Nerves which appeare to run to the capsule were often discovered upon histological investigation t pass by without sending any branches inwards. In fact only one small nerv bundle was observed in the thymic antero-dorsal interlobular septum (fig. 3, and this was traced to the 'thymic plexus' described below, and which is sympathetic plexus. It was suspected that other nerves might be present, autonomic origin, which would tend to make them transparent and difficult t observe in anatomical dissection.

The remainder of the capsule was examined with great care for incomin nerves. Although the vagus nerves were observed to run in close proximity t the thymus dorsally (fig. 4), no branches from them entered the capsul Vital staining and transillumination methods demonstrated thin, faintly stain ing fibres ramifying over the surface of the capsule, but none of these coul be traced inwards. In Ramon y Cajal preparations, these capsular fibres wer traced with difficulty to what might be termed a 'thymic plexus' around th thymic artery, which connected with the plexus mammarius inter surroundin the internal mammary arteries (fig. 3, D). As the plexus mammarius inter con sists of postganglionic sympathetic fibres, the central connexion is therefor that of the cervical sympathetic trunk.

Path of innervation within the gland

Weddell's method showed up large numbers of argyrophil fibres dipping i from the capsular network as seen in fig. 2, E, but owing to conflicting ramifi cations it was not possible to distinguish the two types of fibres and hence t determine the extent of the nervous network. With all the methods utilize only one definite tracing inwards of a nervous pathway was obtained. Had an spinal components contributed to the innervation they would in all probabilit have precipitated the silver salts and, being usually thicker than reticular fibre would have been recognizable, if not traceable. No such fibres were recognize This was further borne out by the fact that in the same series of Ramon Cajal preparations (fig. 2, C and D), extrathymic nerve fibres stained wit success, for example, the nerve fibres in cardiac muscle seen in fig. 3, B, and i the vagus nerve in fig. 3, C.

Serial sections treated after the methods of Ramon y Cajal and of Pines an Majman showed a network of fibres with an affinity for silver around th blood-vessels, as can be seen in fig. 2, A–D. These same fibres were also demon strated in Azan preparations and were seen to be in continuity with the ne work of connective tissue in the meshes of which the thymocytes were foun (fig. 2, F). That they were non-nervous was easily seen as they were of spidery nature and connected directly with the supporting fibres of the inte lobular septa. However, in the case of blood-vessels it was felt that some the fibres showing an affinity for silver were nervous, as a small incomin erve bundle had been identified in the Ramon y Cajal preparation (fig. 3, E). his small bundle would in all probability divide and send out numerous anches along the branches of the blood-vessels although not actually observed do so. The vascular plexuses were so densely stained and their ramifications numerous and closely placed, that it became impossible to distinguish em. Romanes's method provided a reasonable means of masking the con- ective tissue fibres, but it was noted that now no argyrophil fibres of any escription were visible. The reason for this may have been that any fibres esent were solely of postganglionic autonomic derivation and therefore nstainable by present methods, as Romanes (1950) reports that this stain iled to demonstrate postganglionic autonomic nerve fibres.

ltimate nerve endings

No nerve terminals were demonstrated in association with the cells of the ymus. Such as were described by Pines and Majman (1929) were not seen ven in sections prepared after their techniques. The endings which they escribe may be optical illusions, the plane of the section having passed through oints at which the connective tissue fibres bend downwards. Such tangential ctions show fibres with apparent club-shaped endings, and these were prob- ly responsible for the statement of Pines and Majman (1929) that nerve ndings occurred frequently in the medullary tissue. These authors were in ality staining reticulum, but their methods were not even sufficiently success- il to show the full extent of the reticular system. They have mistaken the ner connective tissue fibres for 'neurofibrils of two types'. In fig. 2, B, fine eticular fibres which have been cut tangentially in the wall of the blood-vessel mulate true nerve endings.

In particular, the vicinities of the Hassall's corpuscles and of the reticular ells were carefully searched for nervous elements but none was seen even fter a variety of techniques. This negative neurohistological picture remained e same even in the involuted thymus.

DISCUSSION

In the past the chief hindrance to attributing an endocrine function to the ymus has been the difficulty of ascertaining which cells of the gland could be apable of secreting. There is certainly no evidence based on the innervation hat the thymus performs any secretory function whatsoever, nor, for example, ave endings and networks such as those found in the adrenal (Willard, 935–6) been found. This further evidence of the lack of a secretory function s in agreement with the work of Segaloff and Nelson (1940), Smith and Jones 1940), Burrill and Ivy (1941), Huf and Ripke (1942), Wells, Riddle, and Marvin (1942), Georgiades and Uibenak (1942), and Hoster (1942), who have ignificantly failed to confirm the existence of the hormone formerly suggested y Rowntree, Clark, Hanson, and Steinberg (1935), Bomskov and Siadovic 1940), Biehler, Hanisch, and Wollschitt (1942), and Bomskov, Hoelscher, and von Kaulla (1942). The possibility of incretion cannot, however, be entire ruled out on the present evidence, but if it does occur, it is more likely to l under hormonal than nervous control.

It is interesting to note that during the course of the vital staining exper ments very few signs of phagocytosis by the 'reticular' cells were observe This recalls the statement of Baginski and Borsuk (1939–40) that althoug these cells exhibit a small degree of phagocytosis they do not show athrocytosi

The similarity of the innervation of the thymus to that of a typical lymp node is striking in spite of the fact that true 'lymphatic germ centres' have y to be confirmed in the thymus. It suggests that even if the thymus does n actively produce lymphocytes from 'germ centres' it may play a part in tl regulation of the amount of lymphocytes in circulation at any given time, th acting as a reservoir. This regulation could be controlled by vasodilatator ar vasoconstrictor mechanisms with the possible assistance of thymotropic ho mones entering the gland via the blood-stream. This theory would not co flict with Smith's (1946) observation of lymphocytes leaving the thymus 700-day-old rats by means of fine lymph vessels similar to those described l Sjölander (1915) in human foetuses, nor with the observation of Farr (195 that lymphocytes in the blood-stream which had previously been 'labelle with 3,6,diamino-10-methyl-acridinium chloride in Tyrode were subs quently found in thymic tissue.

Hallion and Morel (1912) by means of a plethysmograph were able observe changes in thymus volume in young dogs after electrical stimulati of the sympathetic cord. It is not clear, however, whether these volume chang were due to vasodilatation of the thymic vessels via vasomotor nerves whether secretory fibres produced an increase in secretion from some cells the gland. This present research would tend to suggest that such an increa in volume is not due to secretory but rather to vascular regulatory activitie

The pattern of the innervation of the thymus does not appear to follow th of the spleen. Utterbach (1944), using silver-oxalic acid techniques on tl spleen of the cat, has described networks of nerve fibres without *bouto terminaux*, but he has not entirely eliminated the possibility of fine connecti tissue fibres staining. The foregoing investigation in the thymus of the mou showed clearly that it is extremely difficult to mask entirely the staining connective tissue fibres with silver particularly in organs which have a den reticulum. It is therefore essential to interpret silver preparations of su organs with extreme caution.

The negative picture produced in the thymus of the mouse by Romanes stain is very suggestive. Romanes (1950) has suggested that the failure of h stain to demonstrate postganglionic autonomic nerve fibres is due to som chemical difference associated with 'the absence of a myelin sheath demo strable by standard techniques' and which is also 'associated with the ability reduce silver chloride'. The failure of the fine 'non-medullated' fibres of tl thymus to stain with silver may, however, be due to even more fundament and complicated chemical differences than the presence or absence of myel heaths, as it is well known that birefringence in polarized light can often lemonstrate fine myelin sheaths in axons previously thought to be non-nedullated when studied by ordinary optical methods. The suggestion is herefore made that the differences obtained by staining with the same rela-ively stable silver solutions may not be due entirely to slight variations in the ctions of the reagents involved, but may also be due to variations in the hemical nature and phase of the axon itself. It is possible that a nerve ending ixed while actively secreting acetylcholine may differ in its staining reactions nd, in particular, its affinity for silver, from a non-active ending or from one vhich is secreting adrenalin.

The conclusion is therefore that as the only demonstrable innervation in the hymus of the mouse is associated with the blood-vessels and appears to be postganglionic autonomic in nature, the thymus gland should be regarded as being more closely related to lymphocytopoietic than to endocrine organs.

It is a pleasure to thank Professor Ritchie of the Zoology Department, University of Edinburgh, for placing the facilities of his department at my disposal for this research; Dr. D. M. Steven for his helpful advice and criti-cism; and Professor N. Millott for reading and criticizing the manuscript. The contents of this paper formed part of a thesis accepted for a Ph.D. degree by the University of Edinburgh, 1951.

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The Thoracic Glands of Hemiptera Heteroptera

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SUMMARY

The thoracic glands of the pyrrhocorid *Dysdercus cingulatus* are described. They originate in the second maxillary segment and grow backwards with the salivary gland system. During post-embryonic development the gland nuclei increase in size but not in numbers. In each instar they enlarge, discharge their secretion, and shrink. The thoracic glands of ten other Heteroptera from eight families are described. All consist of large granular nuclei with little surrounding cytoplasm, most commonly arranged as a pair of elongated ductless glands lying parallel to the salivary ducts and attached distally to either the principal or the accessory glands. The thoracic glands are well supplied with tracheoles, but unlike other insects in which corresponding organs have been described, do not appear to have a nerve supply. The number of nuclei in each gland is surprisingly constant, being about 300 in almost all the species examined, the volume of the gland being greater in the larger insects by increase in the size of individual nuclei. The glands disappear very rapidly after the last moult.

INTRODUCTION

SOMETHING of the thoracic glands of insects has been known since the researches of Lyonet, who in 1762 drew the 'granulated vessels' of a caterpillar and described their innervation and tracheation. He hazarded no guess about their function, and it was left to Toyama, who described the 'hypostigmatic gland' in a paper on the embryology of the silkworm in 1902, to suggest 'the structure of the nucleus . . . assures us that it is a glandular organ'. And there the matter rested until Fukuda (1940) working again with *Bombyx* showed that the thoracic glands were the immediate cause of moulting. Isolated abdomina could be caused to moult by implantation of thoracic glands. Williams (1947) showed that in *Platysamia cecropia* the thoracic glands were in turn dependent upon a hormone secreted by the brain and thus justified Kopeć's original view that a secretion of the brain was responsible for the onset of moulting.

Since then various structures, the morphology of some of which had been known for a long while previously, have been held to be homologous with the thoracic glands of Lepidoptera (see for instance reviews by Williams (1949) and Bodenstein (1953)); but, apart from Lepidoptera, it is only in Orthoptera (Bodenstein, 1951), Diptera (Possompès, 1950), Hemiptera (Wigglesworth, 1952), Neuroptera (Rahm, 1952), and perhaps also Odonata (Deroux-Stralla, 1948) that the function of these organs can be regarded as proved experimentally.

There are very few references to the thoracic glands of Hemiptera. Wigglesworth in 1952 described large nuclei in the fat-body of *Rhodnius* and

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demonstrated experimentally that these represented the thoracic glands Williams (1949) mentions that Edwards (unpublished) had observed thes glands in *Oncopeltus fasciatus*, and Nayar (1953) has seen large nuclei in th fat-body of the Pyrrhocorid *Iphita limbata*, which probably represent th thoracic glands. The 'tracheal organ' of *Nepa* (Hamilton, 1931) is not, a Pflugfelder (1949) suggested, the thoracic gland (see Wigglesworth, 1952)

The present account is primarily a comparative study of the thoracic glands of Hemiptera Heteroptera. *Dysdercus* (Pyrrhocoridae) has been dealt with ir greater detail than the rest, and the embryology and post-embryonic development of the glands in this insect are described.

There seems to be little point in continuing to call this organ the 'prothoracic' gland. It is almost never confined to the prothorax, either here or ir the Lepidoptera, where it was originally so named by Ke in 1930, and it originates in the second maxillary segment. The gland will be here referred to a the 'thoracic gland' throughout.

The salivary glands of Heteroptera, with which the thoracic glands are always associated, are of variable shape but of very constant design. Eacl consists of a thick-walled glandular sac, that will be here referred to as the 'principal gland', from which runs two ducts. One duct goes to the salivar pump. The other runs forward into the head but doubles back to enter a thin-walled sac, here called the 'accessory gland'.

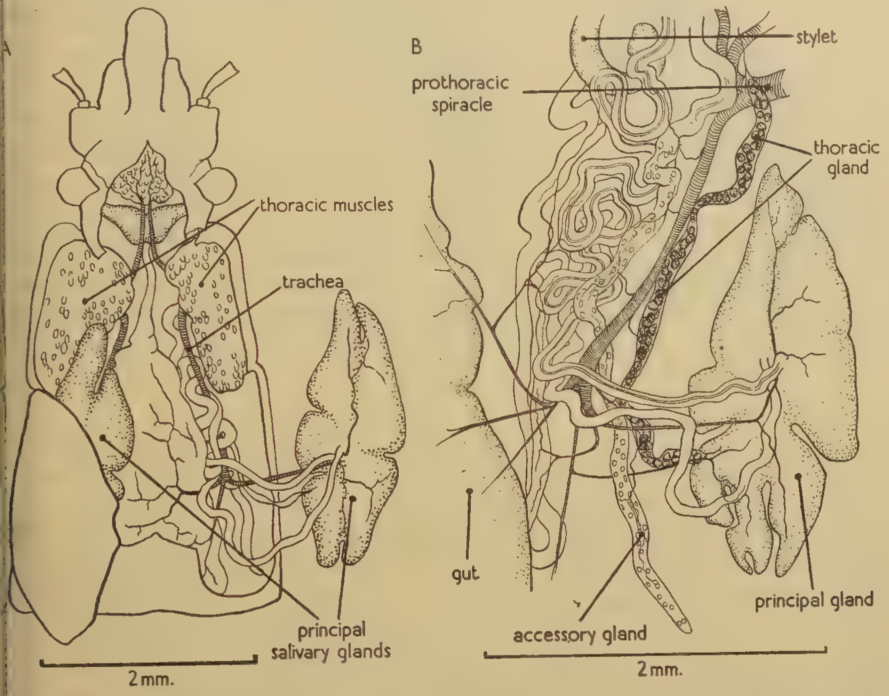
METHODS

Except where otherwise stated the figures and measurements given in the text are from dissections of fifth-stage nymphs. In the other instars the position of the thoracic glands is similar. Dissections were made under insect Ringer after injection with cobalt sulphide (Wigglesworth, 1950). Except where otherwise stated, parts figured were dissected out, fixed in Carnoy, and stained with Delafield's haematoxylin. For material to be sectioned, fixatives and stains were used as specified in the text. For nerves, methylene blue, methylene blue and rongalite, or a silver method (Romanes, 1950) were used.

THE THORACIC GLANDS OF *DYSDERCUS CINGULATUS* F. (PYRRHOCORIDAE)

Demonstration in fifth-stage nymphs

Remove the tergites of the thorax and of the anterior half of the abdomer by slitting along the edges of the body and cutting away the underlyin thoracic muscles. This exposes a network of fat-body through which th salivary glands can be seen. Remove the fat-body and deflect the principa salivary glands outwards (fig. 1, A). The accessory gland and the salivary duc are coiled tubes and may have to be pushed aside to reveal the posterio branch of the prothoracic tracheal system which runs from the prothoraci spiracle to the gut and the principal salivary gland on each side. Suspendec om these tracheae are the thoracic glands. These are best seen if the salivary ands are removed by cutting the tracheae joining them to the gut, severing e prothoracic tracheal system near the spiracle on each side, and removing e head with the salivary gland system and its tracheal supply attached. If is preparation is fixed, stained, and cleared, bits of adhering fat-body can be cked away to show the complete thoracic glands (fig. 1, B).



IG. 1. A, *Dysdercus cingulatus* F. Dorsum of thorax and fat-body removed, right principal salivary gland deflected. B, *Dysdercus*. Salivary and thoracic glands of the right side.

Each gland consists of a rope of cytoplasm almost entirely filled with large, rregularly ovoid nuclei, extending from a point just forward of the prothoracic spiracle to the salivary gland. The nuclei are granular and stain heavily in contrast to the surrounding cytoplasm, which generally stains very little. The gland has an abundant supply of small tracheoles which can readily e demonstrated by injection with cobalt sulphide before dissection (fig. 2, A). have been unable to demonstrate a nerve supply to these glands.

The thoracic glands can also be found in the freshly moulted adult, where heir tracheal supply is still intact and where at first they show no obvious ifferences from those found in fifth-stage nymphs. Within 24 hours (at 27° C.), owever, the nuclei begin to disintegrate (fig. 2, B and C), chromatic droplets ppear, and 2 days after the final moult it is difficult to detect any trace of the lands, which have entirely disappeared.

Behaviour of the glands during post-embryonic development

In each instar the nuclei swell up, discharge their secretion, and shrin again. To show this a supply of nymphs was reared at 27° C. from a sing batch of eggs, and kept until they moulted to the fourth stage. All the

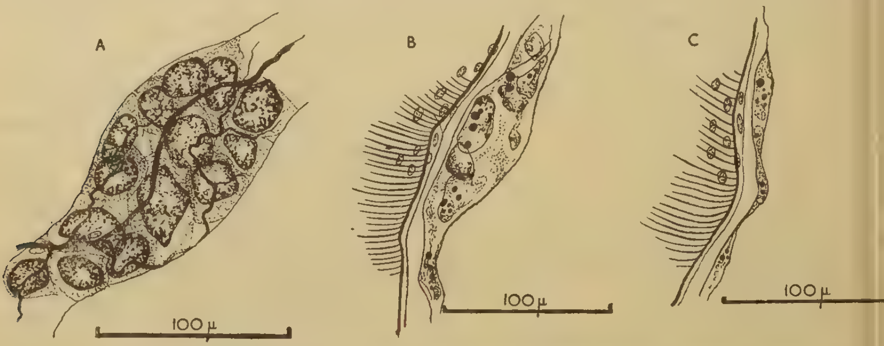


FIG. 2. A, *Dysdercus.* Part of thoracic gland showing tracheal supply. B, 24 hours after fin moult. C, 36 hours after final moult.

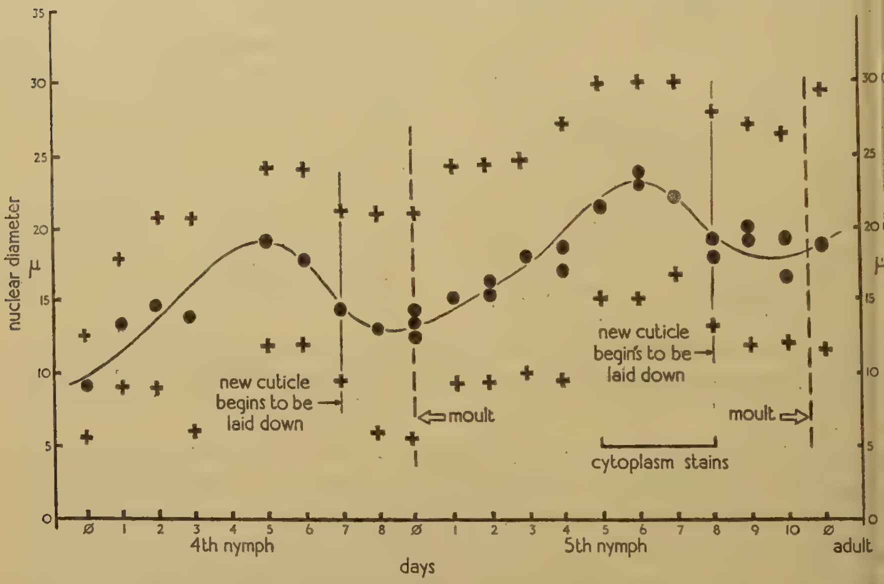


FIG. 3. *Dysdercus.* Cyclic changes in the thoracic gland nuclei. ● Mean diameter of nuclei. + Extreme size limits of nuclei.

nymphs moulted within 48 hours, but only those doing so within the firs 24 hours were selected. Each day a nymph was killed and its thoracic glan cut out, fixed for 5 minutes in Carnoy, stained in Delafield's haematoxyli cleared, and mounted. In this way a complete series (except for 1 day) wa made over the fourth and fifth instars. The six surviving nymphs moulted t adult within 72 hours. A graph (fig. 3) was plotted of day-to-day nuclea ameters, each point being an average of the first twenty nuclei measured. nce nuclear size is very variable even within the same gland, the size range und has also been recorded. It is evident that, at any rate in the fourth and th instars, there is a cycle of nuclear activity. In each instar the nuclei swell ut do not appear to stain more heavily), so that their peak volume may be as uch as five times that at the start of the instar. Peak size is accompanied by discharge of secretion which causes the cytoplasm to stain more darkly than ual and precedes the first appearance of new cuticle by 2 or 3 days. This esumably corresponds to the 'critical period' (Fukuda, 1940; Williams, 52), after which the thoracic glands could theoretically be removed without eventing moulting.

The number of nuclei does not appear to increase during post-embryonic velopment. Counts of the total number of gland nuclei in one of a pair of ch glands in each animal give figures around 300 for all instars. In about ty preparations I have seen no thoracic gland nucleus that appeared to be viding, so that it seems probable that individual nuclei persist without re- acement throughout post-embryonic development. The volume of the gland creases enormously and this is achieved by increase in the size of individual clei.

he origin of the thoracic glands

Toyama (1902) showed that the 'hypostigmatic' (= thoracic) glands of *mbyx* originated from lateral invaginations in the second maxillary segment. nce then, so far as I am able to discover, nobody has traced the origin of ese glands in any other insect. Mellanby (1936) makes no mention of in- ginations into the second maxillary segment of *Rhodnius* other than those stined to become the salivary glands.

In the second maxillary segment of the embryo silkworm there are two pairs ectodermal invaginations, one below and one above the limb on each side. hese become, respectively, the silk glands (= salivary glands of Hemiptera) d the thoracic glands. Subsequent shortening of the head segments and owth forward of the labium takes the openings of the salivary glands for- rd and leaves the thoracic glands hanging into the prothorax from the hind ge of the labium.

The same happens in *Dysdercus*. Eggs were kept at 27° C. At 3½ days g. 4, A) it is possible to make sections showing two pairs of invaginations to the second maxillary segment. One of these pairs is slightly in front of the her and rather larger (fig. 4, B, C, D, and E). This pair almost certainly be- mes the salivary glands. Its position is such that when the appendages of e second maxillary segment fold in and grow forward, the openings will be rried forward and join in the mid-line. The beginning of this process can be en in a 3¾-day embryo (fig. 4, F). The second pair of openings, nearer the id-line of the animal, remains at the hind end of the labial segment. On the urth day the embryo shortens, moves forward, and rolls back upon itself that it comes to rest facing in the opposite direction and is now ventral

surface uppermost. I have not been able to follow the fate of the two pairs
invaginations through this period, but at 5 days (fig. 5, A) groups of cells w
large granular nuclei can be found on each side of the prothorax, where th

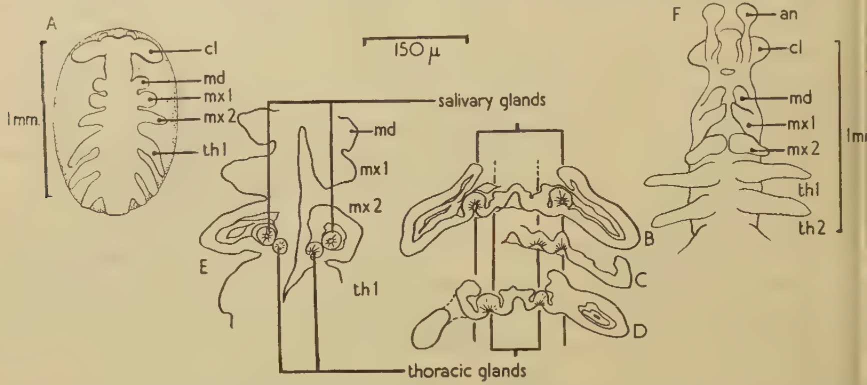


FIG. 4. *Dysdercus.* A, embryo at 3½ days. B, C, D, transverse sections of 3½-day embryc
20-μ intervals. E, sagittal section of 3½-day embryo. F, embryo at 3¾ days. Abbreviatio
an, antenna; cl, cephalic lobe; lb, labrum; md, mandible; mx1, maxilla; mx2, labiu
th1, th2, legs.

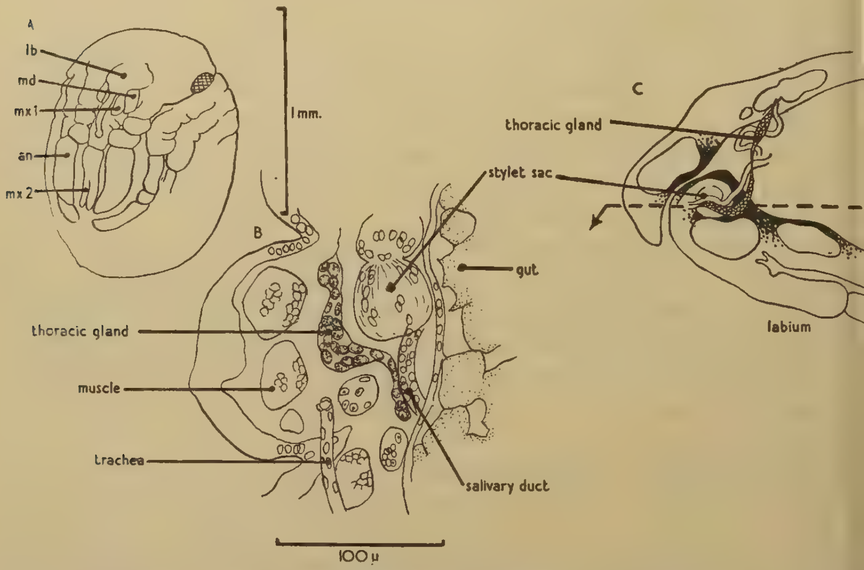


FIG. 5. *Dysdercus,* 5 days old. A, appearance of embryo. B, sagittal section through
thoracic segment. C, reconstruction to show position of section B. Abbreviations as fig

appear to be attached to the last head segment (fig. 5, B). Fig. 5, C, shows
position of this section in a reconstruction based on transverse and lon
tudinal sections made at this stage. That these groups of nuclei are in fact
thoracic glands can be traced from their subsequent history. The gland nu
lie beside the salivary ducts which at this stage are still far forward and

the sides of the embryo over the yolk mass. The salivary glands grow pidly backwards and appear to take the strings of large nuclei with them, til at 6 days (fig. 6, A and B) the gland is very similar in position to that in the st-embryonic stages. It is still attached to the hind edge of the head capsule g. 6, B). The nuclei now have a typical thoracic gland appearance and are uch larger than the salivary duct nuclei beside them (fig. 6, C). On hatching e insect is very full of yolk and passes the first instar without feeding. uring this time the salivary glands extend backwards into the thorax as the lk is absorbed, and the thoracic glands, distally attached to the salivary ands, grow back with them.

The Thoracic Glands in Other Hemiptera

ymnocerata

1. Lygaeidae. *Oncopeltus fasciatus* Dall.

The thoracic glands may be demonstrated in exactly the same way as those *Dysdercus*. As in *Dysdercus*, the glands are attached proximally to a point ear the hind edge of the head capsule, and distally to the principal salivary and. They consist of thin strands of cytoplasm with large (20 μ) nuclei. nlike *Dysdercus*, these are not associated with the prothoracic tracheal stem, but attach to the accessory salivary gland for the greater part of their ngth. The gland nuclei appear to undergo a cyclic development; as in *ysdercus*, and disappear entirely in the adult within 72 hours of the final oult (at 27° C.).

2. Hydrometridae. *Gerris lacustris* L.

Because of the extensive leg muscles, the other thoracic structures tend to e crammed together down the middle of the animal. The greater part of the oracic gland, which is here a less well-defined structure than in most Heterptera, is attached to the accessory gland. It is easiest to demonstrate as llows: Dissect the nymph from the side, removing the mesothoracic leg and leural tergites to reveal the leg muscles, tracheae, and spiracles. Remove the g muscles together with the mesothoracic spiracle and tracheae attached to . This leaves a layer of fat-body overlying the coiled stylets. Strip this off. ix and stain without removal from the body. The thoracic glands are rather iffuse, consisting of very large nuclei (30 μ) mostly grouped along the accesory gland, which can be seen behind the stylets (fig. 7).

3. Reduviidae. *Rhodnius prolixus* Stal. *Triatoma infestans* Klug.

The thoracic glands of *Rhodnius* have been described by Wigglesworth 1952). The gland in *Triatoma* is just like that of *Rhodnius*, the large nuclei of he gland being embedded in the inner lobe of the fat-body, parallel to the alivary duct. These nuclei have a very good tracheal supply and appear to emain in a resting phase when the insect is unfed.

4. Cimicidae. *Cimex lectularius* L.

To demonstrate the thoracic glands dissect off the tergites covering t thorax and the anterior half of the abdomen. The principal salivary glands

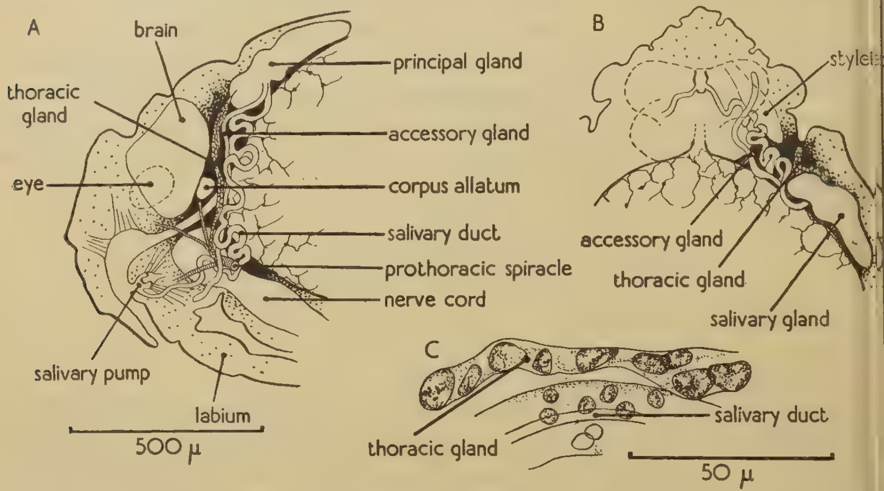


Fig. 6. *Dysdercus* embryo. 6 days old. A, longitudinal reconstruction. B, sagittal reconstru tion. C, parts of the thoracic and salivary glands. Abbreviations as fig. 4.

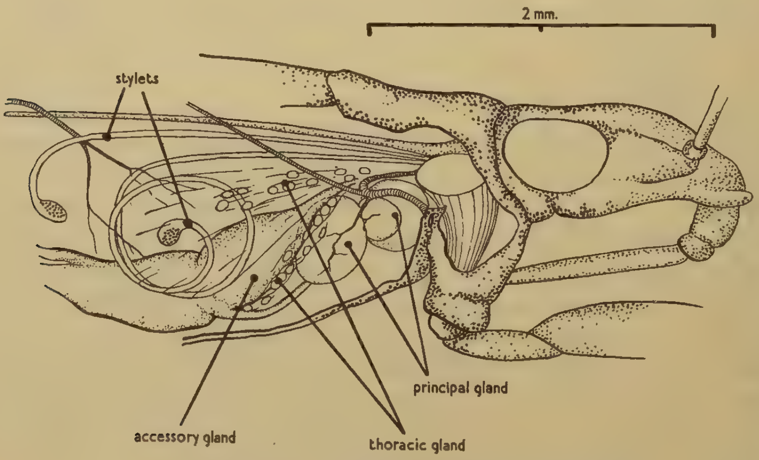


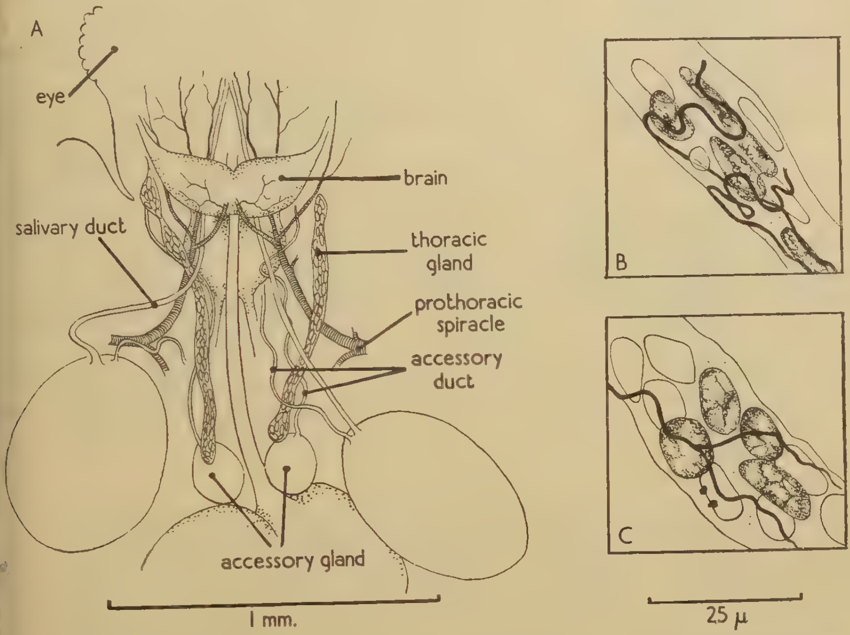
Fig. 7. *Gerris lacustris* L. Dissection from the right side.

reddish bladders that can be seen through the fat-body. Strip the fat-bo away, and deflect the principal glands carefully to reveal the accessory glan which lie on the gut just where the oesophagus broadens out into the crop. F and stain *in situ*. The thoracic glands run forwards from the accessory glan to the sides of the brain (fig. 8, A). As in *Rhodnius* or *Triatoma* the nuclei of t thoracic glands may be in a resting phase. In this event they are long and th

g. 8, B), but still well supplied with tracheoles. In a fed *Cimex* the nuclei large (fig. 8, C).

yptocerata

The thoracic glands of Cryptocerata are much less variable in structure an those of the Gymnocerata here considered, being always attached to the ccessory gland or its duct.



IG. 8. *Cimex lectularius* L. A, salivary and thoracic glands. B, detail of gland in resting phase, showing tracheoles. C, gland in active phase.

5. NEPIDAE. *Nepa cinerea* L. *Ranatra linearis* L.

Nepa. Dissect off the top of the head and the thoracic tergites, cut the horacic muscles, and deflect the principal salivary glands to reveal the thoracic lands attached distally to the accessory glands (fig. 9, B). The forward ends f the thoracic glands attach near the hind edges of the head capsule. Nuclei 5 μ or rather less, subangular and with large nucleoli, are arranged in a single ow for the greater part of the length of the gland. The gland decays within 2 hours of the final moult.

Ranatra. Like *Nepa*, but because the salivary gland system is enormously longated owing to the odd shape of the animal, the thoracic gland may extend nto the abdomen in the later instars (fig. 9, A, is of a third-instar nymph). uclei 15 μ diameter in third-, 22 μ in fourth-instar nymphs.

6. NAUCORIDAE. *Naucoris cimicoides* L.

Dissect as *Nepa*. The principal glands tend to break up rather easily, but

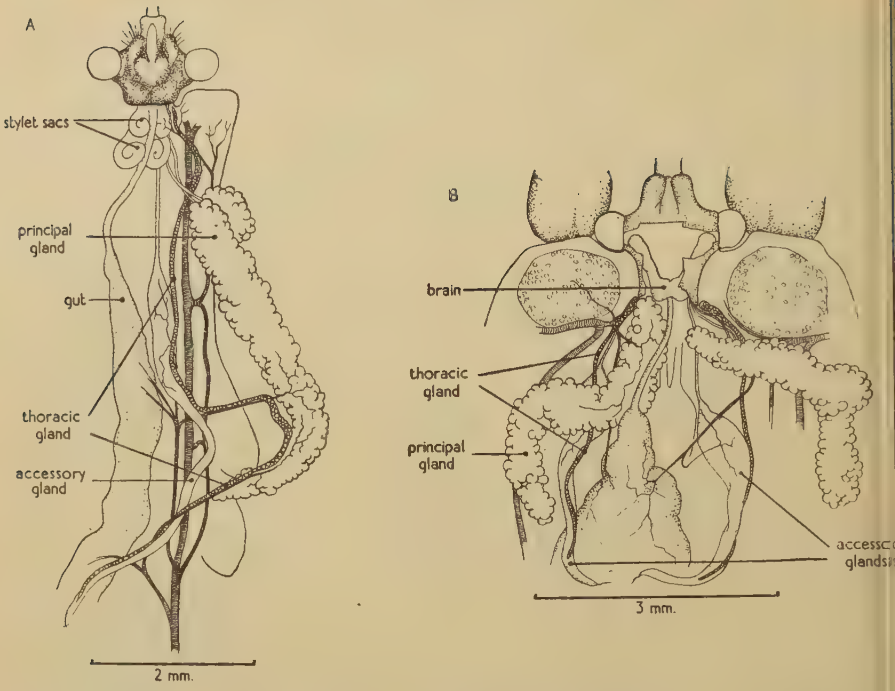


FIG. 9. A, *Ranatra linearis* L. Salivary and thoracic glands of the right side. Third-inst nymph. B, *Nepa cinerea* L. Salivary and thoracic glands.

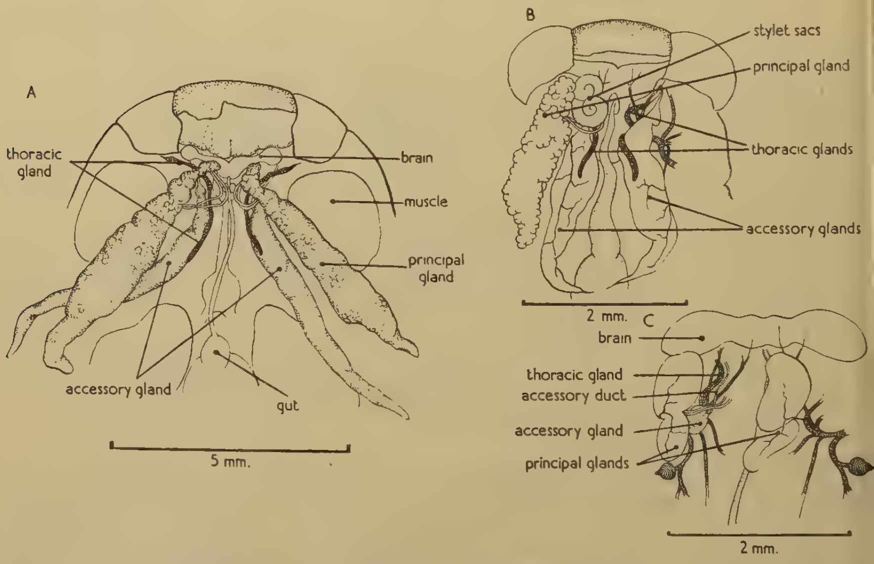


FIG. 10. A, *Naucoris cimicoides* L. Salivary and thoracic glands. B, *Notonecta glauca* Salivary and thoracic glands of a fourth-instar nymph. The principal salivary gland on t right side has been cut out. C, *Sigara striata* L. Salivary and thoracic glands. The princip gland on the left side is deflected to show the accessory and thoracic glands.

is does not matter since the thoracic glands are firmly attached to the cessory glands for most of their length (fig. 10, A). Nuclei of the gland are rge (30μ) granular and oval, but may be so tightly packed as to become subngular. There is a prominent nucleolus.

7. Notonectidae. *Notonecta glauca* L.

Dissect as *Nepa*. The figure (fig. 10, B) is drawn from a fourth-stage nymph. he thoracic gland nuclei have prominent darkly staining nucleoli. Size of the uclei 16μ in fourth-, 20μ in fifth-stage nymphs. The gland degenerates in he adult within 60 hours of the final moult at a temperature of about 20° C.

8. Corixidae. *Corixa punctata* (Illig.) (= *C. geoffroyi* Leach). *Sigara triata* L.

These two are exactly alike in structure in so far as the thoracic glands and eighbouring structures are concerned. Dissect off the top of the head and horax, and deflect the principal salivary glands to reveal the accessory glands, which are thin-walled sacs lying immediately beneath them (fig. 10, C). arallel with the accessory duct on either side is a compact rope of cells. These re the thoracic glands. The nuclei appear to be more coarsely granular in *Sigara* than in most of the species studied.

Discussion

In all the species considered in this report, the thoracic glands are associated with the salivary gland system, being either attached to the accessory gland (Cryptocerata, *Gerris*, and *Cimex*) or running parallel with this. In *Oncopeltus* and *Dysdercus* the distal end of the gland attaches to the principal gland, and n *Rhodnius* and *Triatoma* the gland is sunk into the fat-body, which lies parallel to the salivary duct. It is possible that the thoracic glands are attached to the salivary gland system fortuitously, as a result of parallel growth along the line of least resistance. If this were so, it is difficult to account for the fact that there is always a terminal attachment between the thoracic and salivary gland systems. The thoracic glands might equally well become attached to the gut, or to the tracheal system as in caterpillars. They never are. Probably, since the two systems originate side by side, there is from the first an attachment between them, so that the final form of the glands is dependent upon the structures to which they become attached early in development. As the salivary glands grow back they stretch the thoracic glands with them. If this did not happen, the glands would remain as cell masses on the floor of the hind end of the head capsule, in the position typical of 'ventral glands' in other insects.

The number of nuclei in the thoracic gland of Heteroptera does not appear to increase during post-embryonic development. Actual counts of the number of nuclei in every instar have only been made in *Dysdercus*, but counts of nuclei in two or more instars of several of the other species considered indicate that the same holds good throughout. A detailed search for mitotic figures in serial sections through the intermoult cycle was not made. Nevertheless, because the number of nuclei remains small and constant, and because neith a dividing nor a decaying nucleus was found in any preparation, it seems fair assume that little or no division takes place. In this respect the thoracic glan of Hemiptera resembles the ring gland of Diptera rather than, for instanc the thoracic glands of Orthoptera, in which Scharrer (1948) has shown th mitoses undoubtedly take place during the intermoult period. Measuremen of gland nuclei are necessarily only an approximation. It is probable that cyclic development takes place in other species as it does in *Dysdercus*, that a measurement made from an insect killed at an unknown stage in i cycle may represent a peak or a minimal value. Nevertheless, in the twelv species here considered it is approximately true to say that the bigger th insect, the larger its thoracic gland nuclei. *Corixa* and *Sigara* illustrate th well. The two are almost identical in structure, but *Corixa* is 10 mm. long an *Sigara* 5 mm. The average diameter of the nuclei in the two specimens o *Corixa* was 17μ, in *Sigara* 12μ. Measurements made from other species *Naucoris*, *Triatoma* 30μ; *Ranatra* (fourth nymph), *Nepa*, and *Rhodnius* 25μ *Notonecta*, *Oncopeltus*, and *Dysdercus* all about 20μ; and *Cimex* not more tha 15μ. *Gerris* has few but unusually large nuclei (30μ). (All measurements wer from Carnoy-fixed material.) The number of gland nuclei is about 300 (i each of a pair of glands in every insect) in almost all the species here con sidered. Thus the nuclear volume of the thoracic glands bears the same re lationship to the total volume of the insect in all these species. Moreover, i any one species the volume of the gland nuclei increases logarithmically, a does the total body volume of the insect, from one instar to the next. In on series of measurements from nymphs of *Dysdercus*, for example, the averag volume of thoracic gland nuclei in mid-cycle was 12, 45, 152, 468, and 1,048μ^3 in instars 1, 2, 3, 4, and 5 respectively. Wigglesworth (1937) showed that a fourth- or fifth-stage nymph of *Rhodnius* grafted on to an adult *Cimex* would cause the latter to moult, so that there is no reason to believe that the thoracic gland hormone is any more specific than that of the brain that excites it (Wigglesworth, 1936; Williams, 1946). This being so, the steady relation between nuclear volume and the total volume of the insect implies that the concentration of thoracic secretion needed to induce moulting is the same in all instars and in all these species.

In a continuously feeding insect like *Dysdercus* or *Oncopeltus* there is a very regular cycle of nuclear changes, which persists if the insect is starved after moulting. In the intermittent feeders like *Rhodnius*, *Triatoma*, and *Cimex* the cycle is triggered by feeding (Wigglesworth, 1952), and the nuclei remain in a resting phase until this occurs. Rehm (1950) has shown a cyclic activity of the neurosecretory cells in the brain of *Ephestia*. Presumably this is accompanied by a release of the brain hormone that stimulates the thoracic glands, and presumably it would be possible to demonstrate such a cycle in *Dysdercus* preceding the cycle of thoracic gland development.

In other insects the thoracic glands have an abundant nerve supply (Lee, 1948; Scharrer, 1948). Wigglesworth (1952) was unable to demonstrate a nerve supply to the thoracic glands of *Rhodnius*. In dissections of the Hemiptera here considered no nerves were observed. Methylene blue and silver methods were tried. Since in most of these insects the thoracic glands are elongate structures, often with only a single row of nuclei and frequently hanging freely in the body cavity, nerves should have been readily visible if present. None was seen.

CONCLUSIONS

A. *Gross structure of the gland*

In all the species considered in this report, the paired thoracic glands are:

1. Present in all nymphal instars but absent in the adult, disappearing very rapidly after the last moult.
2. Associated with the salivary gland system and typically attached to the accessory gland. The position in two species of the same family is almost identical even when the animals are of very different shape, but detail differs from one family to the next.
3. Well supplied with tracheoles, particularly in *Rhodnius* and *Triatoma*, where the nuclei are lying on the fat-body. The tracheal supply can be traced to the prothoracic and/or mesothoracic spiracles, but detail differs enormously.
4. Without nerve supply.

B. *Origin*

In *Dysdercus* the glands arise as a pair of ectodermal invaginations into the second maxillary segment, and thereafter grow backwards in company with the salivary glands. But for this their position would be very similar to that of the 'ventral glands' found in other insects.

C. *The gland nuclei*

1. The thoracic glands of Heteroptera consist of strings of large subangular or ovoid nuclei with little cytoplasm.
2. The nuclei are coarsely granular and generally have a nucleolus which stains more darkly than the rest.
3. The number of nuclei is variable, but generally of about 300 in each of a pair of glands in each insect. This number remains unchanged throughout post-embryonic development, individual nuclei persisting without replacement or division.
4. In general the larger the volume of a species, the larger will be its thoracic gland nuclei.

I should like to thank Professor Wigglesworth for his great help and encouragement during this work, which was done whilst in receipt of a grant from the Department of Industrial and Scientific Research.

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The Structure of the Corpus Cardiacum of *Locusta migratoria*

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(*From the Department of Zoology and Comparative Anatomy, Oxford*)

With one plate (fig. 1)

SUMMARY

The corpus cardiacum of *Locusta* consists of a syncytium containing nuclei of two sizes. The most obvious cytoplasmic inclusions are spheroid bodies, mostly about $0{\cdot}6\,\mu$ in diameter, which are easily seen in the living tissue, especially by phase-contrast microscopy. They do not appear in routine microscopical preparations, but are blackened by the classical 'Golgi' techniques. They give positive reactions for phospholipines. The cytoplasm also contains very numerous minute granules, which appear to be mitochondria.

INTRODUCTION

WHILE the structure and physiology of the corpora allata of insects are fairly well known, those of the corpora cardiaca have received comparatively little attention. Recent researches on the neurosecretory cells of the insect brain tend to suggest that the corpora cardiaca have important roles to play in the endocrine system of the insect (Scharrer, 1952; Thomsen, 1952).

Ontogenetically the corpora cardiaca must be considered as ganglia (Hanstrom, 1948), but their histology shows that they function as secretory organs (review by Hanstrom, 1941).

The orthopteran corpora cardiaca have been studied by de Lerma (1933, 1937), Pflugfelder (1937), Cazal and Guerrier (1946), and Cazal (1948). The organs are variously described as being syncytial, or as consisting of nervous and secretory cells or chromophobe and chromophil cells, with a considerable amount of eosinophil and fuchsinophil secretion. Cazal and Guerrier (1946) record the finding of numerous granular mitochondria and Cazal (1948) mentions 'dictyosomes' also in the chromophil cells. The presence of chromaffin granules and the diphenolic nature of the secretion have been reported by Cameron (1953).

MATERIAL AND METHODS

Adults belonging to both sexes of *Locusta migratoria* were kindly supplied by the Anti-Locust Research Centre, London. Three batches of two dozen each were obtained and studied.

In dissections, the pronotum and the vertex of the head of the live locust are cut away and after the animal has been pinned in a dish of saline (0·8 per

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cent. sodium chloride with 0·2 per cent. of 10 per cent. calcium chloride), the region behind the brain is stretched. When the large transverse tracheal trunk behind the brain is cut, the corpora cardiaca come into view as bluish-white elongated bodies, intimately connected to the anterior end of the aorta. The whole tissue is cut out carefully as one piece and transferred either to fresh saline for the examination of live tissue, or else to a fixative. The tissue in saline remains without any noticeable structural change for about an hour.

The following methods were used in the investigation of the minute structure and composition of the glands.

Fresh tissues. Freshly dissected corpora cardiaca were examined in saline under the ordinary, phase-contrast, and polarizing microscopes. The following dyes were used supravitally: neutral red, methylene blue, janus black, and dahlia violet. Vital staining by injection of the dyes was not successful.

Whole mounts. Whole mounts of the glands were made by fixation in 10 per cent. formalin and staining in Mayer's acid haemalum.

Routine sections. Sections of material fixed in Bouin's fluid, Zenker's fluid, and 10 per cent. formalin were stained in Heidenhain's iron haematoxylin.

Methods for showing cell boundaries

(*a*) Herxheimer's Giemsa-tannin method (1915).

(*b*) Recklinghausen's silver nitrate method (1862) for intercellular substances, adapted as follows. Freshly dissected corpora cardiaca were washed in distilled water for 1 minute and then treated in 0·25 per cent. silver nitrate in the dark for 6 minutes. They were then washed in distilled water, mounted on a slide in distilled water, and pressed slightly with a clip; the preparation was exposed to light until brown.

(*c*) Smith's Lyons blue/picric acid method (1912).

For mitochondria. Altmann's acid fuchsin according to Metzner's method (1928) after fixation in osmium tetroxide solution and postchroming.

Classical 'Golgi' techniques

(*a*) Weigl's Mann-Kopsch method (1910).

(*b*) Kolatchev's method (1916).

(*c*) Aoyama's method (1929).

For spheroids and lipochondria

(*a*) Baker's Sudan black method (1944) after fixation in formaldehyde-saline, formaldehyde-calcium, or Champy's fluid.

(*b*) Thomas's modification (1948) of the above for paraffin sections.

(*c*) Thomas's method of studying 'Golgi elements' by treatment in 1 per cent. osmium tetroxide and examination from time to time (1947).

Histochemical methods. Of the many tests tried, the following were the most useful in throwing light on the chemical composition of the structures studied.

(*a*) Baker's acid haematein and pyridine extraction tests for phospholipines (1946).

(*b*) Cain's Nile blue method (1947) for acidic lipides.

(*c*) Cain's plasmal reaction (1949).

(*d*) Baker and Jordan's unpublished modification of the pyronin/methyl green method for nucleic acids.

(*e*) Lison's chromaffine test; the Vulpian reaction; Sevki's method; the hexamine silver method of Gomori; all these for chromaffin and argentaffin granules (Pearse, 1953).

(*f*) Gomori's chrome alum/haematoxylin-phloxine method (1941) for lipofuscins.

(*g*) Schmorl's method (Pearse, 1953) for lipofuscins.

(*h*) Schultze's method for cholesterol (see Pearse, 1953).

RESULTS

Nuclei (see fig. 1, A). The numerous nuclei were especially clearly seen in the thin, flattened parts of the living gland. Most of them were spherical or subspherical, and measured on the average 8 μ in diameter in life. Scattered in the gland, but especially in the vicinity of the nerves, were larger, ovoid nuclei, measuring about 12 μ in greater diameter, on the average. The smaller nuclei contain much chromatin and stain dark blue with pyronin/methyl green. The larger nuclei contain much less chromatin, which appears to be distributed in the form of threads staining pale blue with pyronin/methyl green. The two kinds of nuclei are shown in fig. 1.

Ground-cytoplasm. By phase-contrast this appears to be slightly heterogeneous in life, with faint striations. It stains pale pink with pyronin/methyl green.

Cells or syncytium? Cell-limits could not be seen during life by ordinary or phase-contrast microscopy. None of the methods intended to show cell-limits in fixed preparations succeeded in doing so in the corpus cardiacum, though Smith's Lyons blue/picric acid method (1912) was very successful in showing them in various other tissues. It appears that the corpus cardiacum of *Locusta* is a true syncytium.

Spheroids. The most obvious cytoplasmic inclusions are spherical or subspherical objects, which are easily seen in life, especially by phase-contrast microscopy (see fig. 1, B). The majority are about 0·6 μ in diameter, though a fairly large proportion range up to about twice this diameter (fig. 1, D), and some even to 2 μ. They appear bluish and are easily distinguished from the fat-droplets that sometimes get loose from the fat-body and become entangled in the surface of the gland, for the latter are yellow and always larger.

The spheroids show a perfectly black rim and pale interior under positive phase-contrast, as though they were provided with a cortex of high refractive index (fig. 1, B).

Supravital staining with dahlia violet at 1:10,000 colours the rims of the spheroids bluish-violet and their centres red or reddish-violet. The rim appears to be of slightly uneven thickness.

The other vital dyes tried did not stain the spheroids. Strangely enough 0·01 per cent. neutral red is lethal to the syncytium, for the nuclei become strongly coloured in less than 15 minutes. The dye was tried at 0·003 per cent., at which concentration the nuclei were unaffected, but so also were the spheroids.

In classical Golgi preparations, made by the methods of Weigl, Kolatchev, and Aoyama, the spheroids became enveloped in a deposit of osmium or silver. In Weigl preparations they showed a clear interior and a thick black rim (much thicker than the rim seen by phase-contrast or after vital staining by dahlia). In some cases two or three spheroids were artificially joined together by a deposit of osmium. The most life-like appearance was given by the Kolatchev technique, post-osmication being restricted to 4 days at 34° C. In Aoyama preparations the spheroids usually appear to be blackened all through (fig. 1, c), though sometimes the silvering is restricted to their outer edges. Those who give the name of 'Golgi apparatus' to cytoplasmic inclusions that are not seen in routine microscopical preparations but are blackened by the classical Golgi techniques, will apply it to the spheroids of the corpus cardiacum.

The process of blackening by osmium could be well studied by treating the freshly dissected glands with 1 per cent. osmium tetroxide and examining at intervals. This method of Thomas's showed the gradual blackening of the edges of the spheroids, leading to the ultimate picture seen in the classical Golgi preparations. Recklinghausen's method also proved useful, for reduced silver appeared round the spheroids and displayed them very clearly.

When the organ was fixed in formaldehyde-calcium and coloured with Sudan black, dirty blue-black patches were seen in the cytoplasm. The sudanophil material presumably represented the spheroids.

The spheroids reacted positively to Baker's acid haematein test, but negatively after pyridine-extraction. They therefore may be taken to contain phospholipine. They are coloured blue by Cain's Nile blue method, a result that would be expected if they contained phospholipine. They also reacted positively to Cain's version of the plasmal reaction.

Tests for cholesterol, chromaffin material, and glycogen gave negative results.

The spheroids showed no birefringence between crossed polaroids.

It seems probable that the spheroids represent the endocrine secretion of the gland.

Fig. 1 (plate). Photomicrographs of the corpus cardiacum of *Locusta migratoria*.

A, a pyronine/methyl green preparation, showing the two kinds of nuclei.

B, a fresh preparation in saline, showing the spheroids, which here appear in optical section as small rings. (Positive phase-contrast.)

C, an Aoyama preparation, showing the spheroids blackened by silver.

D, a fresh preparation in saline, showing several of the minute granules. Most of the minute granules are considerably smaller than this and are in continual Brownian movement, so that they cannot be photographed. (Positive phase-contrast.)

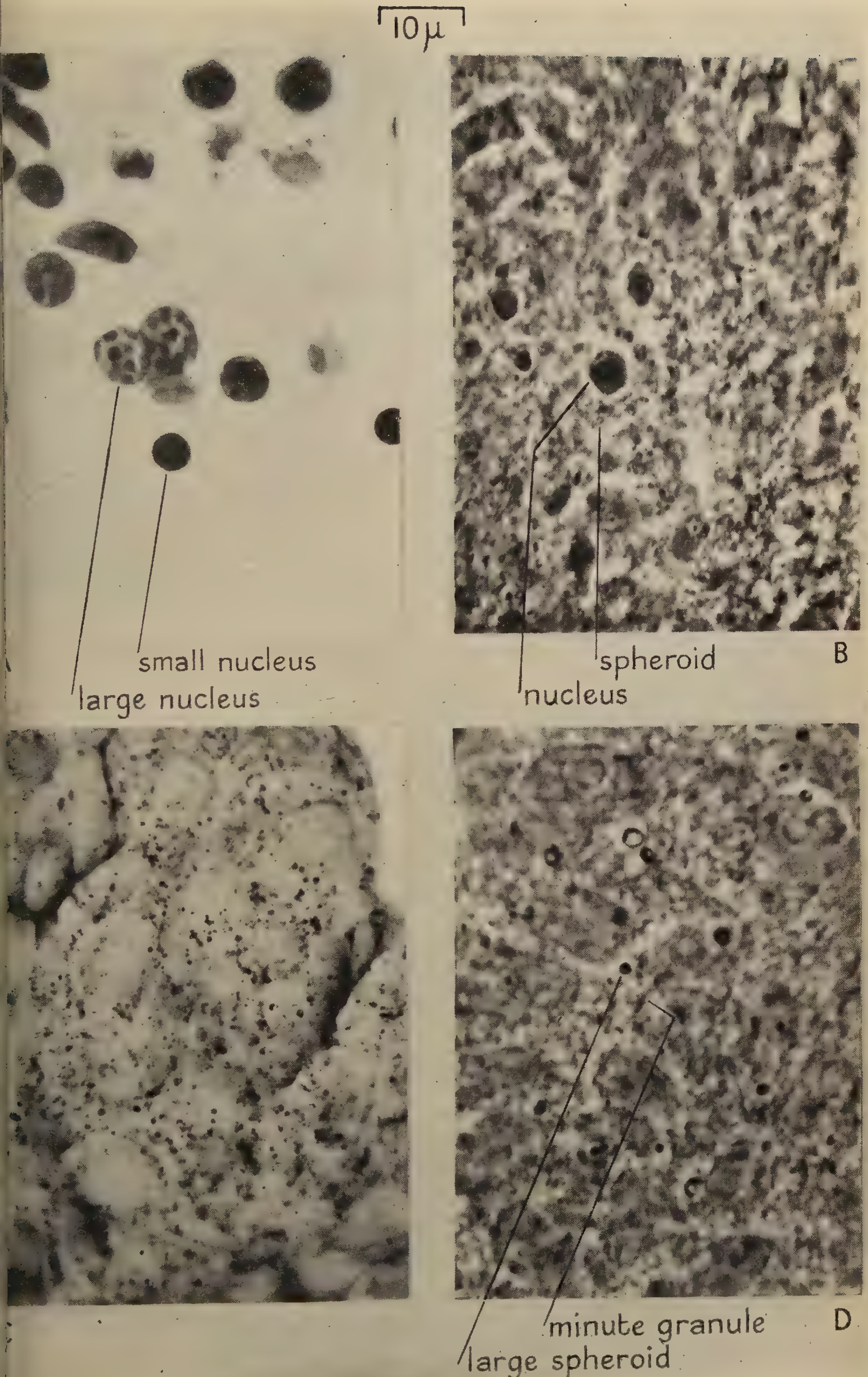

FIG. 1

K. K. NAYAR

Minute granules. The cytoplasm contains a large number of very minute granules, too small to be accurately measured. Some are larger than others. In life they are in active Brownian movement, and this, combined with their very small size, makes it difficult to photograph them in life. The larger ones, however, are sometimes immobile, and several of these are shown in fig. 1, D. When a single granule is accurately focused with phase-contrast, it appears black. These minute granules are stained bluish-violet by dahlia. They were not coloured by the other vital dyes tried, but, although unstained, they were more easily seen by ordinary microscopy after treatment with neutral red at 0·003 per cent. They were found not to be birefringent.

When the gland is fixed in a mitochondrial fixative and stained by Metzner's method, the cytoplasm is seen to contain a large number of minute red granules. These are presumably mitochondria, and it seems likely that they are the same as the minute granules seen in life.

Miscellaneous histochemical notes. Lison's test showed scattered chromaffin granules in the cytoplasm, but negative results were obtained with the Vulpian test and Gomori's hexamine silver method.

The chrome alum/haematoxylin-phloxine method of Gomori showed no positive reaction for lipofuscins in the gland, but in the vicinity of the nerves the Schmorl method revealed blue granules.

The work was done under the guidance of Dr. J. R. Baker, who took a keen interest in the study. I am grateful to him for his constant help and encouragement. I am thankful to him and to Miss B. M. Jordan for making the photomicrographs, and to the latter for teaching me some of the techniques. I am obliged to Mr. H. K. Pusey for suggesting Smith's method of staining cell-boundaries and for giving me the prepared stain, and to Drs. P. C. J. Brunet, W. G. B. Casselman, A. L. Roque, and Mr. S. A. Shafiq for helpful discussions.

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The Mechanism of Proboscis Movement in *Arenicola*

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SUMMARY

The mechanism of proboscis movement is analysed in detail in *Arenicola marina* L. and *A. ecaudata* Johnston, and discussed in relation to the properties of the hydrostatic skeleton.

Proboscis activity is based on the following cycle of movements in both species.

Stage I. The circular muscles of the body-wall and buccal mass contract; the head narrows and lengthens.

Stage IIa. The circular muscles of the mouth and buccal mass relax; the gular membrane (or 'first diaphragm' of previous authors) contracts; the mouth opens and the buccal mass emerges.

Stage IIb. The longitudinal muscles of the buccal mass and body-wall contract; the head shortens and widens and the pharynx emerges.

Stage III. As Stage I.

The two species differ anatomically and in their hydrostatic relationships. In *ecaudata*, the forward movement of body-fluid which extrudes and distends the proboscis is largely due to the contraction of the gular membrane and septal pouches. In *marina*, the essential mechanism is the relaxation of the oral region which allows the general coelomic pressure to extrude the proboscis. The gular membrane of *marina* contracts as that of *ecaudata* does, but its anatomy is different and it appears to be a degenerating structure as far as proboscis extrusion is concerned.

Withdrawal of the proboscis may occur while the head is still shortening and widening in Stage II*b*, or while it is lengthening and narrowing in Stage III. The proboscis is used both in feeding and in burrowing; in the latter case nothing enters through the mouth; the difference is largely caused by variation in the timing of withdrawal relative to the 3-stage cycle.

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PROBOSCIS activity in a lugworm is a highly intricate and flexible performance, yet it is brought about by a relatively simple muscular system acting on a hydrostatic skeleton. Sometimes the whole proboscis is everted, sometimes only a part, and the form of the extruded organ varies to some extent from time to time. In *Arenicola marina*, and probably in the other

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species, there are at least two functionally distinct types of proboscis activity for the organ is used both in feeding and in burrowing, and in the latter cas nothing enters through the mouth (Wells, 1944). The limitations of the hydrostatic skeleton have been emphasized by several authors, and the followin study was undertaken in the hope of finding out how so much can be don with what appears to be a very simple instrument.

Two species were used—*A. marina* L. and *A. ecaudata* Johnston. The differ somewhat in the anatomy of the proboscis apparatus and also, as wi be shown, in its physiological mechanism.

The Anatomy of the Proboscis Apparatus

Only the essential points are summarized below. Fuller details were published elsewhere (Wells, 1950, 1952). Diagrams of horizontal and media sagittal sections of the two species are shown in fig. 1. The horizontal section bring out the underlying plan of the proboscis apparatus more clearly tha the sagittal, because of certain complications which appear in the media plane.

The head is the region extending forwards from the anterior margin of th first chaetigerous annulus (Ch. I). Its form is very variable—e.g. it can b pulled back nearly into a transverse disk in *marina*—but in moderately relaxe specimens it is bluntly conical in *marina* and more elongated and cylindrica in *ecaudata*. Its body-wall has circular and longitudinal muscle layers, and n other muscles (except for certain localized specializations such as the metastomial muscle, which directs the mouth backwards when the worm is abou to turn in its burrow). The body-wall continues into the proboscis at th mouth.

The proboscis is the eversible part of the gut. It consists of (*a*) a bucca mass (bucc. ma.) with large papillae; these are sucker-shaped in *ecaudata* bu tooth-like in *marina*; (*b*) a pharynx (phar.) with numerous fine papillae (*c*) a short post-pharynx (post-phar.) with coarser, blunt papillae. The non-eversible oesophagus (oes.) follows after the post-pharynx. All of these regions have circular and longitudinal muscle fibres.

The gut is suspended from the body-wall by two obliquely transvers sheets, the retractor sheath (ret. sh.) and the gular membrane (gul. memb. this is the 'first diaphragm' of previous authors). These sheets divide th space between gut and body-wall into three distinct cavities, the head coelo (h. coel.) in front of the retractor sheath, the paraoesophageal cavity (paraoes cav.) between the two sheets, and the general coelom (gen. coel.) behind th gular membrane. The retractor sheath and gular membrane probably represent a single septum which has been split into two sheets by the appearanc within it of a non-coelomic space, the paraoesophageal cavity (Wells, 1952) The septum was originally situated at the anterior boundary of the firs chaetigerous annulus, but, as the sagittal sections show, its insertion on th body-wall is now somewhat oblique, and is carried forwards mid-dorsally a

V-shaped peak to a point about half-way between the prostomium (prost.) nd the first chaetigerous annulus.

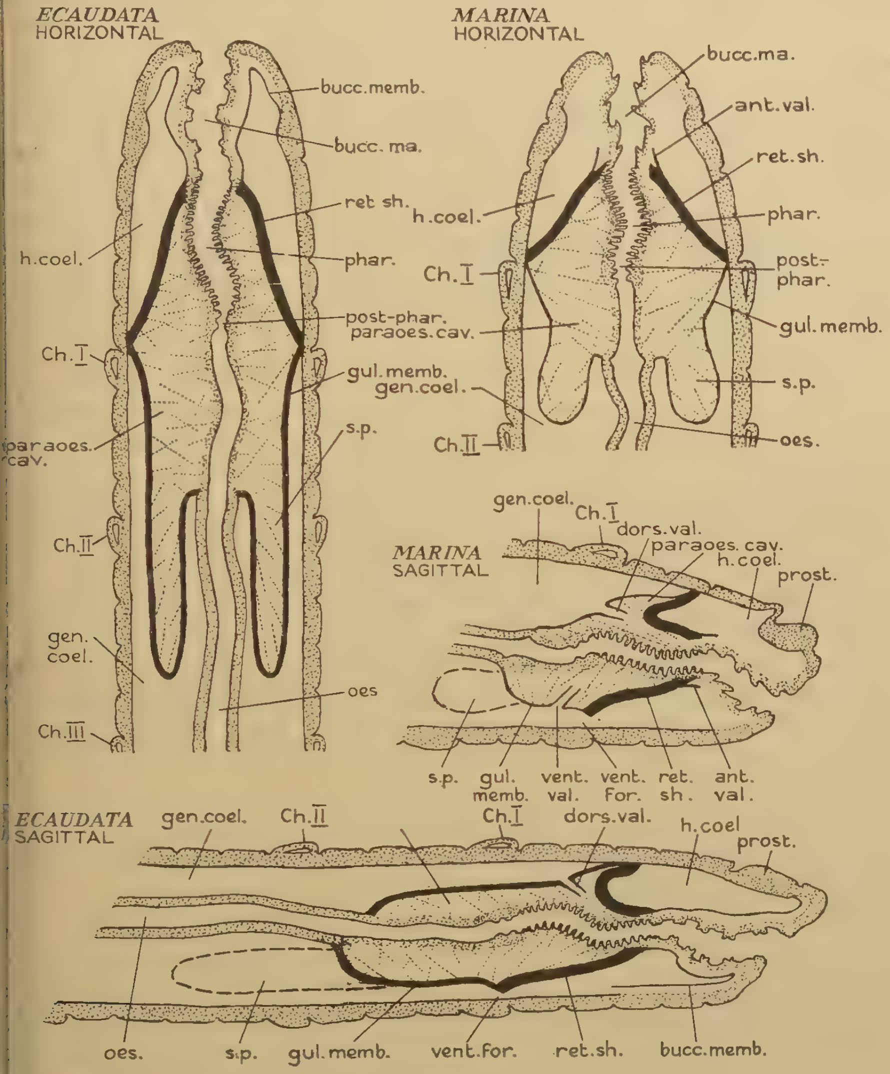


FIG. 1. Diagrams of the structure of the proboscis apparatus. Lettering as on p. 270.

The retractor sheath runs to the junction of pharynx and buccal mass. Its musculature is exclusively radial.

The gular membrane runs to the oesophagus and is carried backwards latero-ventrally into a pair of septal pouches (s.p.). In *ecaudata* the gular membrane is very massive and provided with both circular and radial muscles; its union with the oesophagus lies far behind that with the body-wall so that

its capacity is great. In *marina*, on the other hand, it is much less stout and ha
radial muscles only; its general form is that of a rather flat cone and its sept
pouches are relatively small, so that its capacity is less than in *ecaudata*.

The head coelom communicates with the general coelom in the media
plane by means of the ventral foramen (vent. for.), through which the nerv
cord passes. This foramen can presumably be closed by contraction of th
muscle columns of the retractor sheath and gular membrane which reach th
body-wall just beside it. In *ecaudata* it is comparatively small and is the on
communication between head and general coelom. In *marina* it is larger an
there are a number of minute additional openings between the two coelom
all round the line of junction of the retractor sheath and gular membrane wit
the body-wall; the importance of these openings is however doubtful, an
they may be regarded, from a functional point of view, simply as extension
of the ventral foramen.

The paraoesophageal cavity is crossed by numerous radial muscle strand
running from the retractor sheath and gular membrane to the gut (drawn
dotted lines in fig. 1). In *ecaudata* it is completely cut off from the oth
cavities except at the dorsal valve (dors. val.)—a narrow descending mid
dorsal fold of the gular membrane, so constructed that fluid can readily flo
from the general coelom into the paraoesophageal cavity but not in the revers
direction. In *marina* the gular membrane has not only a large dorsal valve bu
also a functionally similar ventral valve (vent. val.), and it is tightly bound t
the gut mid-dorsally in such a way that it would offer no obstruction to flui
flowing forwards from the general coelom towards the paraoesophageal cavit
Moreover, the junction of retractor sheath and proboscis is elaborated i
marina to form an anterior valve (ant. val.), through which fluid could readi
flow from the paraoesophageal cavity into the head coelom. All of these valve
have muscle strands in their walls, which might conceivably close them.

The papillae of the proboscis are thickenings of the gut-wall, hollowed b
the great development of the interstitial spaces between the cells. The caviti
of the pharyngeal papillae continue into the paraoesophageal cavity, which
probably itself an enormous interstitial cleft. Those of the buccal mass a
ideally cut off from the head coelom by the peritoneal lining of the latt
cavity. In *ecaudata*, however, the lining has separated away from the rest
the buccal-wall as the buccal membrane (bucc. memb.), and in *marina* it h
disappeared altogether so that there is no barrier between the head coelo
and the cavities of the buccal papillae. These modifications presumably ser
to increase the mobility of the buccal papillae.

Farther back, the general coelom is divided transversely in both speci
by the well-known ‘second and third diaphragms’. The second diaphrag
has a large opening on each side. The third is very thin and extensive a
perforated by a great number of minute openings. Neither of them c
effectively hinder a longitudinal movement of body fluid; so the gener
coelom may be regarded, for our present purpose, as extending uninterrupted
backwards from the gular membrane through many segments of the worm

The stomatogastric nervous system was described by Whitear (1953). It onsists essentially of a diffuse network in the walls of the proboscis and esophagus with a nerve ring surrounding the gut just behind the post-harynx.

NORMAL PROBOSCIS ACTIVITY

The proboscis movements of either species can be conveniently observed three ways. (i) Unstimulated worms are simply watched in a bowl of sea-ater. They show occasional bursts of more or less vigorous proboscis activity. i) Worms are tied across at about the level of the sixth or seventh segment, e hinder part is cut away, and the front part is held still by means of a pin st behind the ligature. Proboscis activity occurs much as in (i) and the estriction of movement facilitates observation with a dissecting microscope. ii) Worms are placed in a large glass funnel filled with sea-water. They sually 'burrow' rapidly and vigorously down the stem.

eneral features of the extrusion cycle

All the various types of proboscis action appear to be modifications of a ngle cycle, which is essentially the same in both species. The cycle can be ivided into three stages, characterized by the movements of the body-wall f the head (fig. 2).

Stage I. The head narrows and lengthens, the mouth remaining closed.

Stage II. The head broadens and shortens and the proboscis is extruded. his process occurs in two more or less distinct steps.

Stage IIa. The mouth opens as the distended buccal mass emerges.

Stage IIb. The body-wall of the oral half of the head shortens; meanwhile e buccal mass shortens quickly and powerfully and the pharynx is thrown ut.

The proboscis is generally withdrawn between Stages II and III, but the xact timing of withdrawal is rather variable, as described in detail below.

Stage III. The mouth closes and the oral region of the head narrows and engthens again. This movement sometimes leads directly on to Stage I of e next extrusion, but often there is an interval with other movements, such s shortening and lengthening of the head, before Stage I reappears.

In vigorous proboscis activity, the body-wall of the first two or three haetigerous segments generally follows that of the head, lengthening and arrowing in Stage I and shortening and broadening in Stage II*b*. However, his reinforcement does not always occur. The cycle is sometimes carried out ith only slight changes in the form of the head, restricted to its oral half; in his case extrusion is often, though not necessarily, incomplete.

Withdrawal of the proboscis begins at the end of Stage II*b*, or even just efore it, while the head is still shortening. It is completed either before or uring Stage III. In the first case, the whole proboscis disappears while the ead is still short and broad and the mouth wide open. If the anterior end appens to be directed towards the observer, he can look into the mouth after ithdrawal and see a vast cavity inside the head, as if the walls of the withdrawn proboscis were pulled backwards and outwards against the body-wall. In th second case, the mouth seems to press on the retiring proboscis, and clos as soon as it disappears from view.

Under natural conditions, the general effect of the whole cycle will be, fi

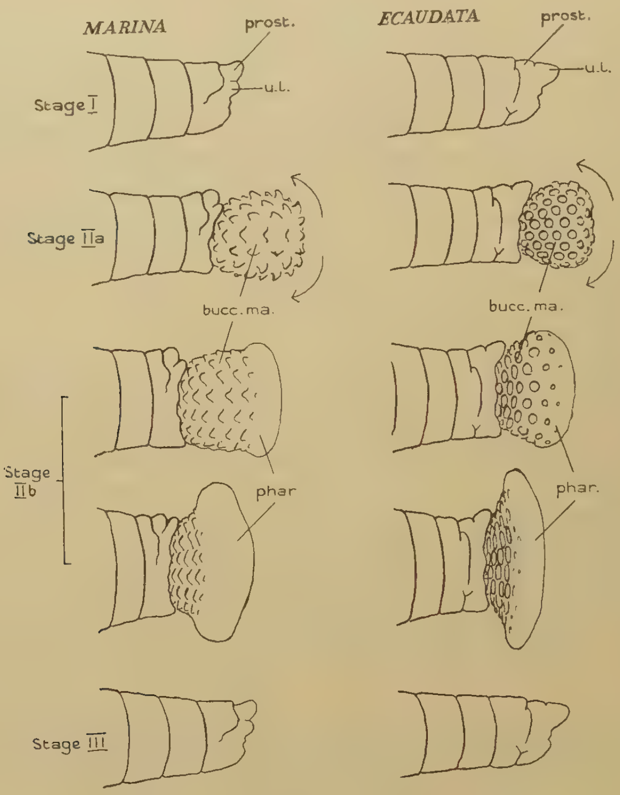


Fig. 2. Sketches in lateral view of normal, vigorous proboscis activity. Lettering as on p. 2
The various stages in the extrusion cycle are described in detail in the text.

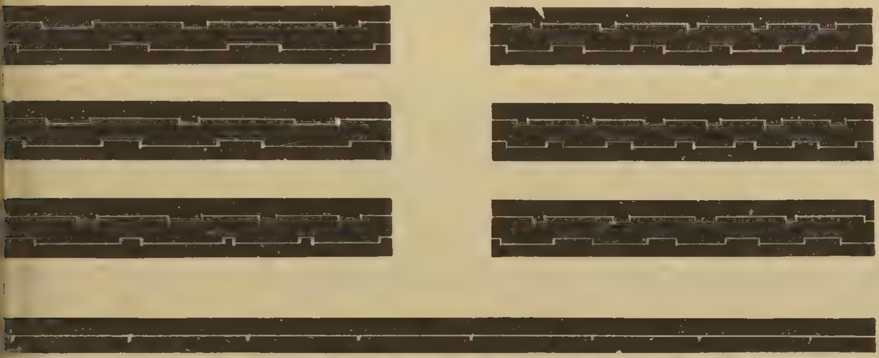
to drive the proboscis forwards into the sand (Stages I and II*a*), and th either to drag the worm forwards or to drag the sand back, as the phary expands laterally and the buccal mass and anterior segments shorten. T variation in the timing of withdrawal relative to Stage III probably underl the distinction between feeding and burrowing mentioned in the openi paragraph of this paper. In the first case, the internal expansion of proboscis, occurring after withdrawal with the mouth still open, would se to suck in sand; in the second, the closure of the mouth around the retiri proboscis would prevent any intake.

The consecutive stages of the cycle may run swiftly and smoothly into e other or be separated by distinct pauses. The whole cycle occurs most rapi in vigorous burrowing, as shown by the tracings in fig. 3. This is pa ecause Stage III of one extrusion leads at once into Stage I of the next under ese conditions; but the tracings show that there is also a general acceleration all stages.

ifferences between the species

Although both species conform to the same cycle of movements, with the me variations, they show certain differences of an essentially anatomical ature. The most striking are as follows.

(i) The emerging buccal mass of *marina* has raised, conical teeth and these ay exert a rasping action on the sand as the organ drives forwards. These eeth flatten down as the buccal mass shortens in Stage II*b*. The function of e buccal suckers of *ecaudata* has still to be elucidated, but they can hardly ct as a rasp.



IG. 3. Records to show the timing of normal extrusion in *A. marina* at 11·0°–13·5° C. The orms were watched and tapping keys connected to the signals were operated by hand. epression of lower signal means exposure of buccal mass, that of upper signal means exposure pharynx. Read from left to right. Time trace in 10-second intervals. The extracts on the left e from spontaneous extrusion outbursts in three isolated anterior ends (worms tied across at bout Chaetigerous Annulus VII, hind part cut away). Those on the right are from three tact worms burrowing vigorously down into the stem of a large glass funnel filled with sea-water.

(ii) The prostomium is separated from the mouth by a strip of body-wall own as the upper lip (fig. 2, u.l.). In *ecaudata*, the prostomium is flat and e upper lip forms a prominent cone in front of it; this cone drives forwards uring Stage I and acts, to some extent, as a spearhead. In *marina*, on the ther hand, the upper lip is very small and when the oral region narrows Stage I it is the prostomium which extends farthest forwards over the outh.

(iii) The pharynx of *marina* is more extensive and more rounded at full xtrusion than that of *ecaudata*. As the pharynx of the latter is extruded, the uccal mass shortens and widens into a flat cone (like a loudspeaker cone) so at the fully exposed pharynx has the form of a flat or slightly domed disk.

THE SEQUENCE OF MUSCULAR CONTRACTIONS

The muscular contractions responsible for proboscis activity cannot safe be inferred from observations on intact worms, or from dissections in whi the hydrostatic conditions still allow normal muscle interactions. In such ci cumstances a change in length of a muscular component may be due eith to a change in its own state of contraction or to a change in pressure caus by the activity of other muscles. Fortunately, the behaviour patterns *Arenicola* remain surprisingly normal after drastic surgical interference wi the working conditions of the muscles. Preparations can be set up in which t tension of each muscular component is virtually independent of the state contraction of the others, and by their use the normal sequence of contractio in the proboscis cycle can be determined.

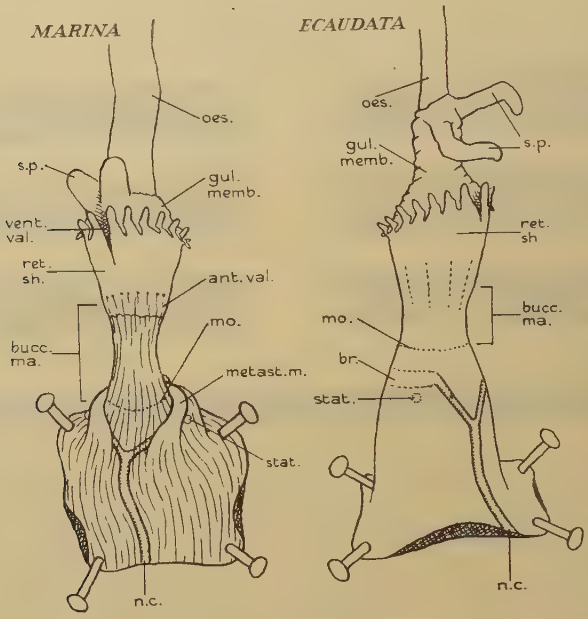


FIG. 4. Sketches of isolated extrovert preparations. Lettering as on p. 270.

The isolated extrovert of A. marina

The so-called isolated extrovert is a preparation consisting of the probos with a piece of oesophagus attached, with or without the brain and the bo wall of the head. It exhibits a regular alternation of periods of rhythmic activ with periods of rest. The intermittent rhythm corresponds with a periodic of proboscis activity often seen in the intact worm, and is due to a pace-ma action of the oesophagus (Wells, 1937). The following results show that correspondence between the dissected preparation and the intact worm extends even to the detailed sequence of muscular contractions in an extrusion cycle.

For the present purpose, the preparation should be set up as follows (fig. 4). The worm is opened by a mid-dorsal cut through the body-wall, which just divides the first chaetigerous annulus but goes no farther forwards. The body-wall of the chaetigerous segments is pinned out laterally, and that of the head is turned forwards by means of two pins pushed outwards through its hinder margin in such a way that the head as a whole invaginates. The various

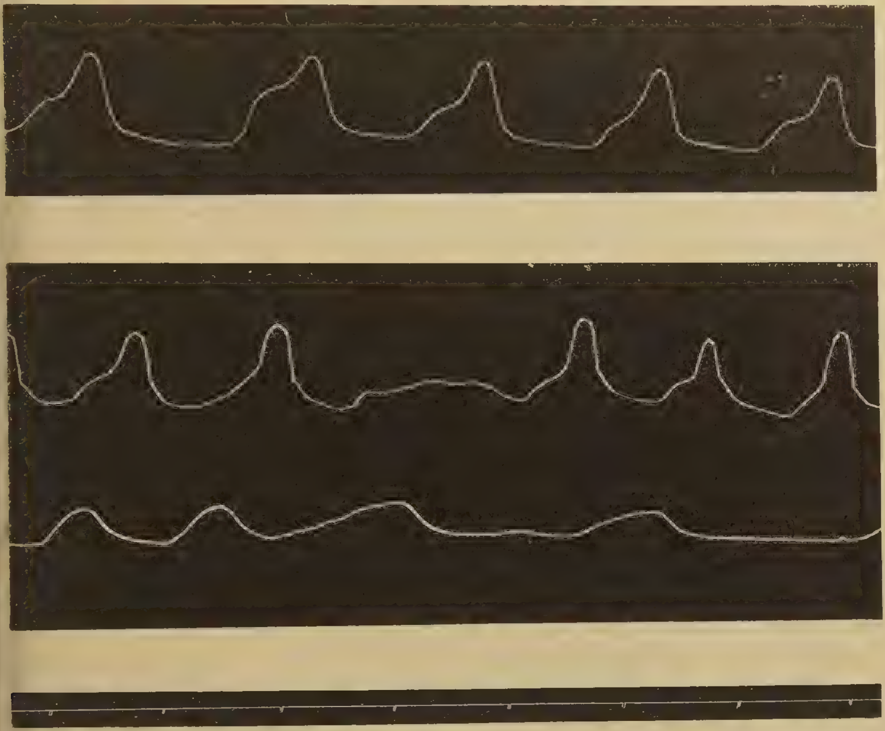


Fig. 5. Records to show the timing of the extrusion cycle in dissected preparations of *A. marina*. Read from left to right. Time trace in 10-second intervals. Frontal writing points. *Above*: part of an activity outburst in an isolated extrovert at 16° C. Dissection as fig. 4. Each peak shows three distinct events: (*a*) shortening due to longitudinal-muscle contraction of buccal mass; (*b*) further shortening due to contraction of retractor sheath; (*c*) sudden lengthening due to circular-muscle contraction of buccal mass and simultaneous relaxation of retractor sheath. Stages II*b* and III of the text correspond respectively with (*a*) and (*c*). *Below*: preparation with simultaneous recording of extrovert (upper line) and circular muscle of body wall just behind the head (lower line) at 12° C. Dissection as in fig. 20 of Wells, 1937. The extract shows part of an activity outburst interrupted by a brief pause between the 3rd and 4th peaks. Behaviour of extrovert as before, except that retractor sheath relaxation is completed after the sharp drop due to circular-muscle contraction of the buccal mass. Body-wall strip contracts in Stage I and relaxes abruptly as the buccal mass shortens in Stage II*b*.

mesenteries and diaphragms posterior to the head are divided to free the gu and the oesophagus is cut across just in front of the tips of the oesophage glands. The joint insertion of the retractor sheath and gular membrane c the body-wall is then carefully divided right round the head, starting at th ventral foramen. The body-wall is cut across transversely at about the lev of the second or third chaetigerous annulus, and the hinder part of the wor is thrown away. The body-wall is pinned to weighted cork in a glass contain so that the movements can be watched in detail and the oesophagus is con nected to a light isotonic recording lever (0·3–0·5 g.).

The preparation shows the usual periods of vigorous rhythmic activi alternating with periods of relative rest. During the vigorous periods, shorten ing generally occurs in two distinct steps, the first due to longitudinal contra tion of the buccal mass and the second to contraction of the retractor sheat (fig. 5, upper extract). Lengthening is very rapid and involves not only the r laxation of the muscles just mentioned, but also an active contraction of th circular muscles of the buccal mass. This contraction passes off during th pause before the next upstroke, the buccal mass and oral region widenin fairly abruptly without change of length. The shortening of the buccal mas which is the first step in the next upstroke, may follow immediately or aft a pause of a second or so. A typical cycle therefore includes the following fou events: (i) shortening of the buccal mass, (ii) shortening of the retract sheath, (iii) lengthening of the retractor sheath, lengthening and narrowin of the buccal mass, (iv) widening of the buccal mass. The last involves n change of length and so does not appear on the tracing.

The body-wall of the head and of the first two or three segments often show longitudinal shortening and circular relaxation during the first step of th upstroke, and the opposite changes during or just after the downstroke. Thes movements can be recorded simultaneously with those of the extrovert b means of a dissection illustrated elsewhere (Wells, 1937, fig. 20). A typic tracing is shown in fig. 5, lower extract.

The timing of the full cycle in the isolated extrovert is about the same a that of the proboscis cycle in intact worms (figs. 3, 5). When the two cycl are compared stage by stage, they are seen to correspond so closely as to lea no room for doubt that they are in fact identical. The contraction of the r tractor sheath, which causes the second step in the shortening of the isolat extrovert, evidently represents withdrawal. This is preceded in the isolat extrovert by vigorous longitudinal contraction of the buccal mass, and a exactly similar movement accompanies normal extrusion of the phary (fig. 2, Stage II*b*). Before this again, the dissected preparation shows a fair abrupt circular-muscle relaxation of the oral region and buccal mass, wh the whole worm shows opening of the mouth and protrusion of the disten and presumably relaxed buccal mass (fig. 2, Stage II*a*). After withdrawal, t isolated extrovert shows a vigorous circular-muscle contraction of the buc mass and often of the head as well, while the intact worm shows narrowi and lengthening of the head (fig. 2, Stage III). Often the isolated extrov passes straight from Stage III of one cycle to Stage I of the next, but occasionally it shows an interval at this point during which the body-wall of the head may shorten and lengthen once or twice without correlated movements of the other parts (fig. 5, lower tracing). Similar variations in intact worms were noted on page 255.

The timing of withdrawal relative to the other events varies in the isolated extrovert just as it does in the intact worm (p. 255). The most usual type of cycle is that illustrated in fig. 5, but sometimes the retractor sheath contraction coincides with Stage III; in this case the preparation shortens as the buccal mass contracts longitudinally in Stage II*b* but undergoes little or no change in length in Stage III (the shortening of the retractor sheath being about equal to the lengthening of the buccal mass); finally, it lengthens again as the retractor sheath relaxes after the end of Stage III. A single preparation may show both types of cycle at different times.

The gular membrane is visible in the isolated extrovert preparation but shows no striking movements, perhaps because the septal pouches are collapsed and the membrane as a whole has lost its normal transverse stretch. A very slight movement of the septal pouches can occasionally be seen to accompany Stage II.

If the light comes from behind the preparation, a movement of the pharynx (which lies within the semi-transparent retractor sheath) can often be seen just after the retractor sheath has shortened. This movement appears to be active and could be due either to the pharynx wall itself or to the radial muscle strands. It probably corresponds with the internal expansion of the pharynx at the end of withdrawal in the intact worm (pp. 255–6).

The isolated extrovert can show independent movements of its various parts—e.g. of the buccal mass or retractor sheath—at times when the integrated extrusion cycle is not taking place.

The isolated extrovert of A. ecaudata

The worms of this species are generally smaller and more difficult to dissect than those of the last, and the buccal mass (whose contractions are responsible for a large part of the length changes of the *marina* extrovert) is relatively short and weak (fig. 4). The powerful gular membrane collapses into a sheath around the oesophagus during dissection and gives only very slight movements. The vigour of the preparation is therefore disappointing, and it lacks the striking intermittent rhythmicity of *marina* (Wells and Albrecht, 1951*b*). However, if the *ecaudata* extroverts are carefully watched, they can often be seen to carry out a cycle identical with that of the *marina* preparations. The retractor sheath may shorten either before or during Stage III. The long collapsed septal pouches show a slight but unmistakable downward bending movement which is evidently due to an active contraction, either of their own musculature or of that of the gular membrane. The bend appears at the beginning of Stage II and passes off as the retractor sheath contracts; it therefore corresponds in timing with the normal contraction of the gular membrane and septal pouches (p. 264).

THE ROLE OF FLUID PRESSURE

Proboscis extrusion obviously depends on the pressure conditions inside the worm. A distinction must be drawn between the pressure in the general coelom and that in front of the gular membrane, because the form of the membrane allows its contraction to bear on the fluid in the paraoesophageal cavity. On the other hand, the head coelom and paraoesophageal cavity are separated only by the retractor sheath. The general form of the sheath, whose musculature is exclusively radial, suggests that no important difference in pressure can exist between these two 'head cavities' except perhaps when it contracts at withdrawal.

The pressure in the general coelom

If a worm of either species is suddenly ligatured at about the level of the sixth chaetigerous annulus (the knot being pulled tight at the moment of proboscis extrusion) and the part behind the ligature is cut away, the anterior end can still extrude and withdraw its proboscis in a vigorous and normal manner. The ligature prevents loss of body-fluid and the body-wall retains its usual taut appearance. If, instead of being ligatured, the worm is simply cut across at the same level, the body-wall between the cut and the head rapidly collapses as the pressure in the general coelom falls to that outside. The effect of pressure equalization on proboscis activity is different in the two species.

A transected *ecaudata* can still extrude its whole proboscis perfectly normally, and will generally continue to do so at intervals for half an hour or longer. The extrusion mechanism is therefore independent of a raised pressure in the general coelom. However, the performance gradually becomes less effective. After an hour or two, the worm can only extrude the buccal mass and that not fully distended, although it still makes strenuous efforts at extrusion (as judged by the changes in shape of the head) and can respond by instant and vigorous shortening to a touch or to vibration of the bench. The gradual weakening of the proboscis mechanism is due, as shown in the next section, to a leaking away of fluid from the paraoesophageal cavity through some permeable part of its wall. Such losses can be made good from the general coelom in the intact worm, and the function of the dorsal valve in *ecaudata* is probably to allow replenishment of the fluid content of the paraoesophageal cavity as leakage occurs.

A transected *marina* is unable, from the first moment, to inflate the pharynx. It carries out all the muscular acts of the extrusion cycle, in normal sequence, but ineffectively. The buccal mass appears in Stage II*a*, but not fully inflated. It shortens and widens in Stage II*b* but the pharynx is generally glimpsed as a crumpled body in the middle of the buccal mass, if it is seen at all; at the utmost, there is a slight inflation round the rim where the buccal mass and pharynx join. This species evidently depends on fluid delivered under pressure from the general coelom for full extrusion.

The role of fluid pressure in *marina* is made clearer by the experiment shown in fig. 6. A longitudinal dorsal cut is made in the body-wall in segments vii–viii, and the gut, which immediately bulges out, is divided between oesophagus and stomach. The oesophagus is pushed well forward into the anterior end with a seeker handle, and a cannula (internal diameter 5–7 mm.) is inserted into the body-cavity and tied into position as shown. The hinder part of the worm is cut away. The cannula is connected to a wide tube (internal diameter 32 mm.) and suspended in a bath of sea-water. The pressure in the general coelom can now be adjusted at will by simply varying the water levels inside and outside the wide tube. The movements of the worm hardly affect the level of the sea-water in the wide tube; so the device holds the coelomic pressure nearly constant at any chosen value.

If the internal and external levels are equalized, the preparation behaves like a transected worm; it extrudes the incompletely distended buccal mass but not the pharynx. If the internal level is set below the external, the body-wall collapses and blocks the opening of the cannula, the result being the same as before. If the internal level is raised above the external, perfectly normal extrusions occur with distension of the pharynx. Quite small pressures suffice for this; even at 1 cm. sea-water the distension is fair and at anything from 2 to 10 cm. the extrusions are full and normal.

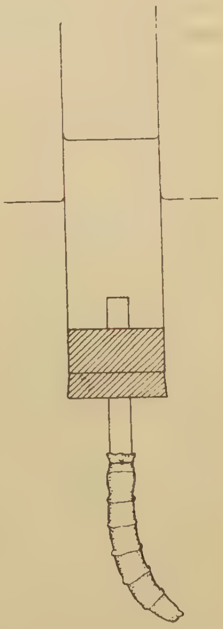


Fig. 6. Experiment to show the effect of varying the pressure in the general coelom on proboscis activity.

At 15 cm. or higher, the body-wall stretches transversely and longitudinally and shows little or no movement during the proboscis cycle; the proboscis can still be extruded and withdrawn but it often appears with a series of curious jerky movements and with an abnormal configuration. This result appears to conflict with the manometric measurements of Chapman and Newell (1947), who found pressures much higher than 15 cm. water in the body-cavity of *A. marina* during proboscis activity. The apparent discrepancy may be due to the artificial conditions of my experiments. If the coelomic pressure is held constant, relaxation of either the circular or the longitudinal muscle layer automatically increases the tension in the fibres of the other, and this reversal of the normal relationships may be expected to upset such regulating mechanisms as the worm possesses.

The results obtained at more moderate pressures show that it is not necessary, for proboscis extrusion, that the coelomic pressure should vary; there is no question of extrusion being caused by a sudden rise in its value. The anterior end apparently contains a releasing mechanism of some kind which allows the pressure in the general coelom to act on the proboscis at the moment of extrusion. The same conclusion was reached by Chapman and Newell (1947), who wrote:

> It was also observed that during movements of the anterior end of the body the proboscis was extruded and withdrawn at irregular intervals, but the movements were not accompanied by any observable rise in pressure of the body fluid. Its extrusion, a rapid movement, appeared to be effected not by any specific rise in pressure but by the release of some catch or trigger mechanism which restrained the organ from eversion during the normal range of working pressures.

The pressure in the head cavities

If a transected worm is further dissected by a mid-dorsal incision running forwards to between the first and second chaetigerous annuli, the divided body-wall is pinned out sideways, and the diaphragms, mesenteries, &c., are removed as far as is necessary to expose the hinder face of the gular membrane, its powers of extrusion are unchanged. An *ecaudata* can still extrude the whole proboscis and a *marina* the buccal mass only.

In the case of *ecaudata*, the movements of the gular membrane can now be watched. The whole membrane and the septal pouches are inflated at the beginning of the cycle, and contract down (the pouches expelling practically the whole of their content) as the proboscis is extruded. They expand again as the proboscis withdraws or a moment afterwards. The gradual weakening noted above in the transected worms now happens much more rapidly—few worms dissected as here described retain the power to extrude the pharynx for more than 5–10 minutes—and one can see that the process is accompanied by a gradual but permanent collapse of the gular membrane and septal pouches. When the preparation is so enfeebled that only the buccal mass can be extruded, the hinder part of the gular membrane forms a close sleeve round the oesophagus and the collapse of the septal pouches is complete. Nevertheless, slight movements of these parts show that they still contract at buccal mass extrusion, and the contraction of the anterior (now nearly transverse) part of the gular membrane pulls the oesophagus forwards and presses on the contents of the head. The power of extrusion can be abolished almost completely at any time by making two wide cuts in the anterior part of the gular membrane, one on each side; the worms then go through the usual configuration changes of the proboscis cycle, but can force out two or three rows of buccal papillae at the most, and often fail to produce anything at all. These observations show that a rise of pressure in the head cavities, produced by contraction of the gular membrane and septal pouches, plays an essential part in extrusion in this species.

In the case of *marina*, one can see, by inspection from behind, that the gular membrane and septal pouches contract rhythmically, once for each extrusion, but unfortunately the general shape of the head is such that the gular membrane and proboscis cannot be watched simultaneously. If the gular membrane is incised on both sides the power of extrusion is almost completely lost, as in *ecaudata*; so probably the imperfect extrusions of the buccal mass seen in a transected *marina* are brought about by contraction of the gular membrane and septal pouches.

DISCUSSION

The sequence of muscular contractions appears to be the same in both species, and is summarized in table 1.

TABLE 1

Stage	*Muscles of body-wall and proboscis*	*Muscles of gular membrane and retractor sheath*	*Result in the intact worm*
I	Circular muscle of body-wall and buccal mass contracts	Gular membrane and retractor sheath relaxed	Head narrows and lengthens
II*a*	Circular muscle of oral region and buccal mass relaxes	Gular membrane contracts	Distended buccal mass emerges
II*b*	Longitudinal muscle of body-wall and buccal mass contracts	No change	Head and buccal mass shorten and widen; pharynx emerges
		Retractor sheath contracts; gular membrane relaxes	Proboscis withdraws
III	Circular muscle of body-wall and buccal mass contracts	Retractor sheath relaxes	Head narrows and lengthens

The first column shows the movements of the body-wall and its continuation, the proboscis wall. These structures have circular and longitudinal muscle layers and as far as can be seen the two layers act reciprocally at any point, with the important exception that in Stage II the relaxation of the circular muscles of the oral region and buccal mass precedes by a more or less definite interval the contraction of their longitudinal muscles. The movements of the buccal mass are always swift and vigorous. Those of the body-wall vary greatly from time to time, in vigour and in the extent of the muscle field over which they spread.

The second column shows the movements of the main 'first septum derivatives', i.e. the gular membrane (with its extension, the septal pouches) and the retractor sheath. These structures have radial muscles only, except for the gular membrane of *ecaudata*, and even here the two muscle layers appear to act synchronously. The timing of the gular membrane contraction shown in the table is based on that of *ecaudata*; that of *marina* is probably the same but for practical reasons it could not be precisely determined. The time of contraction of the retractor sheath varies relative to the body-wall cycle; it may be completed before or after the onset of Stage III. There is some evidence that the radial strands crossing the paraoesophageal cavity contract at the end of the retractor sheath contraction (internal dilation of the proboscis, pp. 255–6; movements of the pharynx region in the isolated extrovert, p. 261).

The third column shows the corresponding changes in configuration of the intact worm (compare fig. 2, p. 256).

The mechanism of extrusion

The contractions of Stage II result in extrusion because of the hydrostatic conditions, and in this respect the two species show important differences.

In *ecaudata*, extrusion can occur in the absence of a raised pressure in the general coelom. The following explanation accounts for the facts. In Stage II*a*, the contraction of the gular membrane and septal pouches raises the pressure in the paraoesophageal cavity and (by displacing the relaxed retractor sheath) in the head coelom. At the same time the mouth opens and the whole of the buccal mass musculature relaxes. The buccal mass is therefore everted and distended. In Stage II*b*, the gular membrane and septal pouches remain contracted (or complete their contraction); the extruded buccal mass and the body-wall of the head shorten, and the result of their contraction is to turn out and inflate the pharynx. This explanation supposes that there is no back-flow through the ventral foramen in Stage II*b*. As the full cycle can occur repeatedly in transected worms, when the pressure in the general coelom is equal to that outside the proboscis, it seems likely that the foramen is closed when the gular membrane contracts. This closure could be brought about by the columns of the membrane which reach the body-wall immediately lateral to the foramen.

In *marina* a raised pressure in the general coelom is necessary for both stages of extrusion to occur normally. In the absence of such pressure, the buccal mass is extruded but not fully distended in Stage II*a*, and there is no eversion of the pharynx in Stage II*b*. The same movements take place as in *ecaudata*; the contraction of the gular membrane and septal pouches contributes to the extrusion of the buccal mass and a longitudinal shortening of the buccal mass and body-wall to that of the pharynx, but a forward flow of fluid from the general coelom into the head cavities normally takes place as well. The fluid can easily pass forwards through the dorsal, ventral, and anterior valves (and perhaps also through the ventral foramen, which is larger in *marina* than in *ecaudata*). The release mechanism probably lies in the mouth, and the following explanation is suggested. In Stage I, the pressures in the various internal cavities are equal and exceed that outside, but extrusion is prevented by the closure of the mouth. In Stage II*a*, the mouth opens and the whole of the buccal musculature relaxes; the buccal mass therefore moves out through the mouth; the gular membrane contracts but its volume displacement is insufficient to distend the buccal mass; the consequent drop in pressure in the head cavities results in a forward flow from the general coelom through the valves. In Stage II*b*, the shortening of the body-wall and buccal mass everts the pharynx; if that organ is relaxed, and if its capacity exceeds the volume-change produced by the shortening, its eversion will be accompanied by a further forward flow.

It will be noted that the above explanation gives no reason why the gular membrane of *marina* should exist at all. The two species most nearly related to *marina* are *A. cristata* Stimpson and *A. loveni* Kinberg, in both of which the septal pouches are much larger and more powerful than in *marina* (Ashworth, 1912, Plates IV, VIII). This fact suggests that the extrusion mechanism of the two species is more like that of *ecaudata*, and that the gular membrane of *marina* is in process of degeneration, at least as far as its participation in extrusion is concerned. The membrane could perhaps be useful in other activities, e.g. in adjusting the arrangement of the withdrawn apparatus or, by raising the pressure in front of its insertion on the oesophagus over that behind, in passing food along the front part of the gut. The latter possibility may be of real importance, as the musculature of the oesophagus is very feebly developed in front of the gular membrane and more powerful behind it.

The mechanism of withdrawal

Withdrawal, in both species, is brought about by contraction of the retractor sheath. The relative timing of this act is variable, and its variation probably underlies the distinction between feeding and burrowing. If withdrawal is completed during Stage II*b*, while the head and buccal mass are still short and broad, the contracted sheath will tend to pull the buccal mass into a wide cylinder lying just inside and parallel to the body-wall. This may well exert a sucking action and draw sand into the mouth, particularly if the radial strands contract at the same time and so dilate the pharynx. The subsequent wave of circular muscle contraction which runs from the mouth along the buccal mass in Stage III will then cause swallowing. If, on the other hand, withdrawal occurs when the head and buccal mass are already narrowing and lengthening, there will not be the same tendency for the lumen of the buccal mass to dilate, and the closure of the mouth around the retiring proboscis will obstruct the intake of sand.

A different explanation of the fact that no sand intake occurs during burrowing was given by MacGinitie and MacGinitie (1949, pp. 201–3):

> *Arenicola* is provided with an eversible proboscis. . . . The outside of this everted organ is well supplied with glands that secrete mucus when the worm is feeding, causing organic matter and sand to stick to it. Upon inversion, the proboscis carries the entrapped material inward to be swallowed. There is a certain amount of selection, since only the finer material and grains of sand adhere to the proboscis, and coarser material is shoved to one side. . . . When *Arenicola* burrows without feeding, very little detritus or sand sticks to the proboscis, indicating that secretion by the mucous glands on the proboscis has been greatly inhibited.

This mechanism could coexist with the one suggested in the present paper.

Proboscis action and the hydrostatic skeleton

It is a striking feature of the proboscis mechanism of *marina*, whose gular membrane is comparatively unimportant, that the first stage of extrusion is largely determined, not by a local contraction, but by a local relaxation. The work is derived from the coelomic pressure, or by drawing a levy from the tonus of the whole of the body-wall musculature. The mechanical principle resembles that of the lever. On the one hand, the act is a vigorous one, and the form of the emerging buccal teeth suggests that they exert a rasping action on the sand at this moment; on the other, the capacity of the extruded proboscis is small compared with that of the coelom, and the power comes from an extensive muscle field whose individual fibres shorten very little.

In this respect, the lugworm exploits a fundamental property of the hydrostatic skeleton, which was emphasized in a recent discussion by Batham and Pantin (1950). If the skeleton of an animal consists of a single reservoir of fluid under pressure, the performance of any act involves the whole of the muscular system, since work is done by tapping the pressure reserve which in turn affects the lengths and tensions of all the muscles acting upon it.

One of the advantages of a skeleton composed of rigid jointed members is that it allows the mechanical isolation of local movements. A vigorously burrowing *Arenicola* does several things at the same time, but each of them affects the pressure conditions in the coelom and so potentially interferes with the rest. The proboscis is extruded and withdrawn; the anterior segments lengthen and shorten; waves of swelling run forwards along the branchiate region and several segments anterior thereto (Just, 1924; von Buddenbrock, 1928); the more anterior chaetigerous annuli, which contain special cavities communicating with the coelom, are first raised and at the same time inflated, and then depressed and deflated (Wells, 1945, 1950). Even a quietly feeding worm does other things as well, though there is a tendency in *marina* for the irrigation waves to be inhibited during the feeding periods (Wells, 1949; Wells and Albrecht, 1951*a*). It is therefore interesting to inquire how an animal with a hydrostatic skeleton can decrease the mutual interference of several acts performed simultaneously.

Three possible ways suggest themselves. The first is to subdivide the body cavity so that the zone indirectly affected by each act is restricted. The second is to integrate the acts so that a dilation at one point is synchronized with a contraction at another. The third is to increase the capacity of the fluid reservoir relative to the volume changes involved in the various acts, so that their effect on the general pressure is minimized. The septa of annelids result in a limited degree of hydrostatic isolation of different parts of the body (Newell, 1950). In *Arenicola*, however, the septa are mostly reduced and only the first, which is elaborated into the retractor sheath and gular membrane, might be of importance in this way. The gular membrane of *ecaudata* can deliver the necessary volume of fluid under a pressure adequate for extrusion, and so allows a partial isolation of extrusion from other events occurring farther back. Although its action could be reinforced by a high pressure in the general coelom, and its forward movement must indirectly affect the pressure relations behind it, its presence is a guarantee against the blocking of extrusion by a sudden drop in coelomic pressure. In *marina*, on the other hand, full extrusion can only be achieved by drawing off fluid from the general coelom and energy from the body-wall musculature. The various movements of a burrowing *marina* are not very rigidly integrated in time, and it is probably significant that this species is relatively broader than *ecaudata* and has a more spacious fluid reservoir. Extrusion of the pharynx is accompanied, in both species, by a shortening of the buccal mass, which may perhaps be regarded as an economy of volume in the fully extruded proboscis.

The hydrostatic skeleton evidently limits the complexity of an animal's behaviour at any moment. On the other hand, it allows a high degree of functional flexibility. There are no fixed muscle antagonists, and a small number of muscular components can be made to perform a variety of acts at different times by varying the combinations in which they contract. This principle was illustrated in the case of the circular and longitudinal layers of the body-wall by Chapman (1950), and is seen also in the proboscis apparatus. The striking difference between feeding and burrowing without intake of sand is apparently due in the main to a variation in the timing of retraction relative to the three stages of the extrusion cycle. The contraction of the gular membrane in Stage II assists extrusion, but, theoretically at least, the same contraction, occurring with the mouth closed, could play a part in swallowing. An *Arenicola* can sometimes be seen to open and close the mouth, and even suck in water, without any extrusion, and this must be ascribed to combinations of muscle actions differing from those of the typical proboscis cycle. A lugworm lacks the workshop of specialized tools with which an arthropod is provided; but it has a number of alternative activity patterns latent in its organization, which allow it to achieve diversity of performance with a comparatively meagre anatomical equipment.

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LIST OF ABBREVIATIONS USED IN THE TEXT-FIGURES

ant. val.	anterior valve.	n.c.	nerve cord.
br.	brain.	oes.	oesophagus.
bucc. ma.	buccal mass.	paraoes. cav.	paraoesophageal cavity.
bucc. memb.	buccal membrane.	phar.	pharynx.
Ch.	chaetigerous annulus.	post-phar.	post-pharynx.
dors. val.	dorsal valve.	prost.	prostomium.
gen. coel.	general coelom.	ret. sh.	retractor sheath.
gul. memb.	gular membrane.	s.p.	septal pouch.
h. coel.	head coelom.	stat.	statocyst.
metast. m.	metastomial muscle.	u.l.	upper lip.
mo.	mouth.	vent. val.	ventral valve.

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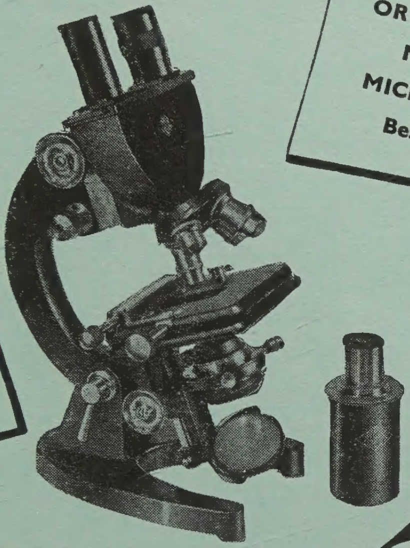

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